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THE INTERACTIONS OF UREAPLASMA UREALYTICUM AND MYCOPLASMA HOMINIS WITH NEISSERIA GONORRHOEAE AND THE ATTACHMENT OF UREAPLASMA UREALYTICUM TO HELA 229 CELLS.

by

(C) MICHELLE JOSEPHINE ALFA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL MICROBIOLOGY

EDMONTON, ALBERTA

SPRING 1986

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THE INTERACTIONS OF UREAPLASMA UREALYTICUM AND MYCOPLASMA HOMINIS WITH NEISSERIA GONORRHOEAE AND THE ATTACHMENT OF UREAPLASMA UREALYTICUM TO HELA 229 CELLS.

Ph.D

SPRING 1986

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Michelle J. Alfa
(Student's signature)

7 Boxwood Cove
(Student's permanent address)

Winnipeg, Manitoba

Date: April 6, 1986

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The Interactions of Ureaplasma urealyticum and Mycoplasma hominis with Neisseria gonorrhoeae and the attachment of Ureaplasma urealyticum to HeLa 229 cells", submitted by Michelle Josephine Alfa in partial fulfilment of the requirements for the degree of Doctor of Philosophy, in Medical Microbiology.

Janet A. Robertson
Supervisor

Michael D. Gabridge

E. Nadon

Carol Cass

Kenneth K. Kay

Glen Armstrong

Date: February 4, 1986/March 19, 1986

THIS THESIS IS DEDICATED TO MY MOTHER AND FATHER, BETTY AND JIM FOUBERT

ABSTRACT

Nongonococcal and gonococcal urethritis are the two most common infectious diseases of adult males. Ureaplasma urealyticum causes approximately one-half of the cases of nongonococcal urethritis, yet is one of the least understood pathogens of the human genital tract. Attachment of the prokaryote to eukaryotic cells is one of the most important stages in many diseases. Although ureaplasmas are known to be closely associated with urethral epithelial cells and persist in the urethra for a long time, there have been few attempts to study the attachment characteristics using in vitro models. There have been many epidemiological studies of gonococcal urethritis, which is caused by Neisseria gonorrhoeae, but little consideration has been given to the interactions of Neisseria gonorrhoeae with other microorganisms in the urethra. Understanding both the interactions of microorganisms and how they remain localized will contribute ultimately to disease prevention. The interactions of the genital mycoplasmas Ureaplasma urealyticum and Mycoplasma hominis with Neisseria gonorrhoeae as well as the attachment of Ureaplasma urealyticum to HeLa 229 cells were examined in this research.

Neisseria gonorrhoeae is frequently isolated on medium containing serum. Although the neisseria cultures are tested for the absence of other bacteria, consideration is

rarely given to the presence of genital mycoplasmas. In this study I demonstrated that approximately one-third of the isolates of Neisseria gonorrhoeae from urethral exudate contained genital mycoplasmas. These genital mycoplasmas persist despite multiple passages of the neisseria on serum-containing media. Indeed, ureaplasmas appeared able to adhere to Neisseria gonorrhoeae. Furthermore, Mycoplasma hominis was associated with alterations in the relative molecular weight of the major outer membrane protein and the auxotype of Neisseria gonorrhoeae auxotype standard strains. To prevent interrelated growth in vitro, serum-free medium should be used for the isolation and propagation of Neisseria gonorrhoeae.

The HeLa cell model was useful for studying the attachment characteristics of ureaplasmas since U. urealyticum strain 27 and strain T960-CX8 attached to this eukaryotic cell line in a time- and inoculum-dependent manner at 36°C and 4°C. However, no viable ureaplasmas were detected 48 hours to 72 hours post-infection therefore, chronic infection could not be studied using this model. Fluorescence, scanning and transmission electron microscopy were used to demonstrate ureaplasmas that had attached to HeLa cells. An ^{125}I-radioassay was evaluated as an alternative to viability assays. Similar attachment characteristics of ureaplasmas were determined by both assays but the ^{125}I-radioassay was more sensitive and rapid than the viability assays. Studies of the ureaplasma

membrane indicated that carbohydrate material which contained glucose and mannose was immunogenic. However, species-specific polyclonal antiserum did not inhibit attachment. Adsorbed horse serum components appeared to have proper orientation to act as adhesins but polyclonal antiserum directed against horse serum did not inhibit attachment of ureaplasmas to HeLa cells. It is possible that attachment of ureaplasmas to eukaryotic cells involves hydrophobic interactions since cholesterol inhibited attachment.

This research describes previously unrecognized effects of the interrelated growth of genital mycoplasmas and Neisseria gonorrhoeae and provides a foundation for further analysis of Ureaplasmas urealyticum attachment to eukaryotic cells.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CCU	colour change unit (i.e. the the inverse of the highest dilution of B broth that changes colour due to growth of ureaplasmas)
CCU_{50}	colour change unit 50% endpoint (i.e. the dilution at which 50% of the samples tested (where the samples are of equal volume) change the colour of B broth due to growth of ureaplasmas)
CFU	colony forming unit
CPE	cytopathic effect
CPM	counts per minute
CUH^-	citrulline-uracil-hypoxanthine requiring
FITC	fluorescein isothyiocyanate conjugated
FM	fluorescence microscopy
GC	gonococcal agar with no horse serum
GCS	gonococcal agar with five percent horse serum
g	gram
GM	genital mycoplasma agar
GU	gonococcal urethritis
H buffer	N-2-hydroxyethyl piperazine-N´-2-ethanesulfonic acid (Hepes) buffer
HRP	horseradish peroxidase
hr(s)	hour(s)
IR	^{125}Iodine radiolabelled
Kv	kilovolts
L	liter

lbs	pounds
LM	light microscopy
M	molar
mA	milliamperes
Mdal	megadalton
MEMS	minimum essential medium supplemented
MEMS-amp	minimum essential medium supplemented with ampicillin
MEMS-gent	minimum essential medium supplemented with gentamicin
µg	microgram
mg	milligram
min(s)	minute(s)
µm	micrometer
mM	millimolar
µl	microliter
ml	milliliter
Mr	relative molecular weight
NGU	nongonococcal urethritis
N.R.	non-requiring
OUH^-	ornithine-uracil-hypoxanthine requiring
P^-	proline-requiring
PBS	phosphate buffered saline
PCU^-	proline-citrulline-uracil requiring
PGU	post-gonococcal urethritis
P.I	major outer membrane protein of Neisseia gonorrhoeae
PMN	polymorphonuclear leukocyte

rpm	revolutions per minute
RT	room temperature
SD	standard deviation
SDS-PAGE	sodiumdodecylsulfate - polyacrylamidegel electrophoresis
Sect.	section
SEM	scanning electron microscopy
TBS	tris-buffered saline
TEM	transmission electron microscopy
TTBS	Tween 20-tris-buffered saline
UV	ultra-violet
V	volts
v/v	volume per volume
w/v	weight per volume

CHAPTER I. INTRODUCTION

Mycoplasmatales are prokaryotes which lack cell walls and are unable to synthesize either mucopeptide polymer or its precursors. This feature separates mycoplasmas from other prokaryotes (Freundt 1955, Taylor-Robinson & Gourlay 1984).

The first mycoplasma was isolated and cultivated on synthetic medium in 1898 by Nocard and Roux during their studies of bovine pleuropneumonia. Dienes and Edsall (1937) were the first to isolate mycoplasmas from humans. Mycoplasmas are now known to be ubiquitous (Table 1) with ninety-five strains recognized in the Class **Mollicutes**, Order **Mycoplasmatales** (Taylor-Robinson & Gourlay 1984). The current taxonomic scheme (Table 1) indicates heterogeneity among this class of microorganisms. All mycoplasmas of established importance in human medicine belong to the Family **Mycoplasmataceae**; these include Mycoplasma pneumoniae, Mycoplasma hominis and Ureaplasma urealyticum. **Mycoplasmataceae** have an obligate requirement for cholesterol (Table 1), which is normally supplied by the addition of serum to the growth medium.

1. Characteristics of genital mycoplasmas

Shepard (1954) isolated the first ureaplasma from a male with non-gonococcal urethritis (NGU). Initially called "tiny-form PPLO" (Shepard 1954), the name Ureaplasma urealyticum was proposed in 1974 (Shepard et al 1974). Comparative studies of ureaplasmas isolated from humans and

Table 1 Taxonomy and properties of **Mycoplasmatales**

Division: **Prokaryotae**
Class: **Mollicutes**

	Current Number of recognized species	Genome Size (10^8 daltons)	Mol% G+C of DNA	Cholesterol requirement	Habitat
Order I. **Mycoplasmatales**					
Family I. **Mycoplasmataceae**					
Genus I. *Mycoplasma*	69	5	23-41	+	Animals
Genus II. *Ureaplasma*	2*	5	27-30	+	Animals
Family II. **Acholeplasmataceae**					
Genus I. *Acholeplasma*	8	10	27-36	-	Animals possibly plants
Family III. **Spiroplasmataceae**					
Genus I. *Spiroplasma*	3	10	25-31	+	Arthropods and plants
Genera of uncertain taxonomic position:					
Genus *Anaeroplasma*	2		29-34	+	Rumens of cattle and sheep
Genus *Thermoplasma*	1	10	46	-	Burning coal refuse piles

* The two recognized species are: *Ureaplasma urealyticum* and *Ureaplasma diversum*.

Excerpt from Razin (1978) and Taylor-Robinson and Gourlay (1984).

from animals indicated that they have significantly different protein profiles (Howard et al 1981, Mouches et al 1981). Howard (1982) later proposed a second species within the ureaplasmas, Ureaplasma diversum, for bovine strains. U. diversum has been adopted in the latest edition of Bergey's Manual of Systematic Bacteriology (Table 1). For the purpose of this thesis, only ureaplasma strains isolated from humans will be considered. The ureaplasmas isolated from humans can be subdivided into at least fourteen serotypes (Robertson & Stemke 1982). The serological identification schemes that have been reported are based on growth inhibition (Black 1970), complement-dependent killing (Lin & Kass 1970), colony fluorescence (Black and Krogsgaard-Jensen 1974) and immunoperoxidase (Quinn et al 1981). For clarity, all serotypes will be designated according to current classification schemes with the designation that was used in a specific reference given in parenthesis.

Ureaplasmas are typically round or coccobacillary in shape (Shepard et al 1974, Robertson et al 1983) and bounded by a single membrane (Black et al 1972a). Evidence indicates that ureaplasmas have an extracellular layer of carbohydrate (Shepard 1967, Robertson & Smook 1976, Smith 1984a). Smith (1984a) reported that lipoglycans account for about 5% of cellular dry weight of the U. urealyticum serotypes 3, 4 and 8 tested. Ureaplasmas have been reported to have cellular diameters that range from 0.33 µm to 0.85 µm (Black et al 1972a, Shepard et al 1974),

witha mean diameter of 0.5 um based on morphometric studies (Robertson et al 1983). U. urealyticum and other mycoplasma cells are the smallest self-replicating organisms known. Under favourable growth conditions the ureaplasma colonies can range in size from 15 µm to 125 µm diameter (Shepard & Masover 1979, Robertson 1982a). The size of the colonies decreases as the total number of colonies increases (Shepard 1967). The colony shapes may be typical "fried-egg" or "cauliflower" formations, depending on the strain and the culture conditions (Manchee & Taylor-Robinson 1969b, Shepard & Masover 1979). Electron microscopic examination of cross-sections through ureaplasma colonies reveals a relatively thin zone of surface growth and a deeper, more central core of growth into the agar (Black & Vinther 1977). The optimal growth temperatures range from 35°C to 37°C (Shepard 1956, Ford 1962). Initial difficulties in growing ureaplasmas were due primarily to the suboptimal pH (e.g. pH 7) of the media. Although a near neutral pH is suitable for most other mycoplasmas, ureaplasmas grow best at pH 6.0 ±0.5 (Shepard & Lunceford 1965). On agar medium, ureaplasmas grow optimally in the presence of 5% to 15% CO_2 (Shepard et al 1974, Robertson 1982a). For growth of ureaplasmas and all other organisms in this family, serum supplementation of media is necessary. For nutrient effectiveness, horse serum at concentrations of 10% to 20% is most commonly used (Shepard & Masover 1979). While unidentified nutritional requirements may exist, two essential factors known to be provided by serum

are: urea (approximately 0.3 mg/ml) and cholesterol (approximately 0.7 mg/ml) (Shepard & Masover 1979). Rottem et al (1971) showed that cholesterol and B-sitosterol satisfy the sterol requirement of ureaplasmas. The cholesterol is incorporated into the ureaplasma membrane without esterification or any other chemical change (Rottem et al 1971).

Although ureaplasmas are differentiated from other mycoplasmas by their ability to hydrolyze urea (Purcell et al 1966, Shepard & Lunceford 1967, Ford & MacDonald 1967), little is known about the role this metabolite plays in the physiology of ureaplasmas. Shepard and Lunceford (1967) reported that when all the urea from horse serum was removed either by dialysis or urease treatment, ureaplasmas did not grow. On the other hand, Masover et al (1974) reported that if allantoin and putrescine were added to dialyzed medium then ureaplasmas could grow in the absence of urea. He suggested that ureaplasmas had a growth requirement for urea and that allantoin and putrescine acted as urea substitutes. Furthermore, they reported that the addition of acetohydroxamic acid (a urease inhibitor) slowed, but did not prevent, the growth of ureaplasmas. As they indicated, the effect of hydroxamic acid inhibition may relate to iron chelation or some other metabolic function that does not involve urease. The minimum concentration of urea needed to support growth of ureaplasmas has been reported as 0.03 mM to 0.15 mM (about 2µg/ml to 10 µg/ml) (Masover et al

1974, Furness & Coles 1975). Masover et al (1976b) observed maximum growth rate of ureaplasmas at 3.2 mM urea, and Kenny and Cartwright (1977) reported that the minimum generation time observed (1.6 hr.) was at 3.2 mM urea. The hydrolysis of urea by the ureaplasma urease leads to the production of ammonium ions and CO_2 (Shepard & Lunceford 1967). The accumulation of ammonium ions causes the pH of the medium to become alkaline (Shepard 1967). The urease activity has been shown by cytochemical studies (Vinther 1976) and cell-fractionation studies (Masover et al 1977a) to be confined to the soluble, cytoplasmic fractions of the organisms. At least three stable urease isoenzyme forms have been distinguished by zymogram banding patterns (Delisle 1977). The urease enzyme of ureaplasmas facilitates easy colony identification by the urease spot test developed by Shepard and Howard (1970).

Ureaplasma urealyticum is not arginolytic or glycolytic (Black 1973) and therefore lacks the mechanisms utilized by other mycoplasmas for adenosine 5'-triphosphate generation (Schimke & Barile 1963, Black 1973). Urea may serve as an energy source. Masover et al (1977a) suggested that urea hydrolysis could lead to formation of an ion gradient across the membrane. The energized membrane might then be coupled to ATP generation through the membrane-bound adenosine triphosphatase. The ability of urease inhibitors to stop growth (Kenny 1983) supports this hypothesis. However, in the absence of conclusive experimental data, the mechanism of energy production in ureaplasmas remains unresolved.

Ureaplasma strains isolated from humans have genome sizes ranging from 4.1×10^8 to 4.8×10^8 daltons (Black et al 1972b) and have a mol% guanine+cytosine ratio of 27.7 to 28.5 (Black et al 1972b, Howard et al 1974).

The results of electrophoretic analysis of ureaplasma cellular proteins (Razin et al 1970, Sayed & Kenny 1980, Mouches et al 1981, Howard et al 1981) showed that ureaplasmas isolated from humans were distinct from other mycoplasmas and from ureaplasmas isolated from animals. Ureaplasmas that had been isolated from humans could be subdivided into two groups based on the SDS-PAGE patterns of whole cells. Serotypes 1,3, and 6 had similar patterns and 2,4,5,7,8,9,10,11, and 12 formed the second group (Mouches et al 1981, Swenson et al 1983). This subdivision into two groups is supported by the results from DNA hybridization (Christiansen et al 1981), restriction endonuclease digestion patterns (Razin et al 1983), and manganese sensitivity (Robertson & Chen 1984). Proteins comprise about two-thirds of the mass of ureaplasma membranes, the balance being mostly lipid (Rottem 1980).

Ureaplasma serotypes 1 to 8 have been shown to produce a soluble β-hemolysin active against guinea pig and rabbit erythrocytes (Black 1973). Only the U. urealyticum serotype standard 3 (designated serotype III by Black) adsorbs guinea pig erythrocytes (Black 1973). Robertson (1982b) reported that although U. urealyticum serotype 3 (strain 27) did hemadsorb, not all of the wild-type isolates that showed the

type 3 determinant hemadsorbed. Manchee and Taylor-Robinson (1969b) reported that agar colonies of six ureaplasma strains tested all tenaciously adsorbed tissue culture-grown HeLa cells. Another property of ureaplasmas that has recently been demonstrated for all fourteen serotypes is IgA protease activity (Robertson et al 1984, Kilian et al 1984).

Mycoplasma hominis was probably the first mycoplasma isolated from humans (Dienes & Edsall 1937). The genome size of M. hominis is 4.5×10^8 daltons (Bak et al 1969). Nucleic acid hybridization studies and restriction endonuclease digestion patterns indicated that M. hominis strains isolated from blood, the urogenital tract and cell cultures formed three distinct clusters of similar M. hominis strains (Barile et al 1983). The mean cellular diameter of M. hominis is 0.42 µm and the mean cell volume (if the shape is taken to be spherical) is 0.038 μm^3 (Robertson et al 1983). M. hominis colonies range in size from 200 µm to 300 µm diameter (Taylor-Robinson and McCormack 1979). Optimal growth of M. hominis occurs when the pH is between 7 to 8 (Rodwell & Mitchell, 1979). M. hominis has been shown to be arginolytic (Schimke & Barile 1963, Pollack 1979). Schimke and Barile (1963) reported that the arginine dehydrolase pathway, consisting of arginine deiminase, ornithine carbamyl transferase and carbamate kinase was found in M. hominis. M. hominis was one of the earliest recognized mycoplasmal cell culture contaminants. Chromosomal aberrations have been observed in

WI-38 cell cultures infected with M. hominis (Paton et al 1967). M. hominis attaches to HeLa, human embryo (Manchee & Taylor-Robinson 1969a), human diploid lung fibroblasts (Kihara et al 1981), Vero, WiDr, and 3T6 (Barile et al 1983) eukaryotic cells. Adsorption to human or guinea pig erythrocytes was difficult to detect (Barile et al 1983).

2. Colonization and association of genital mycoplasmas with diseases of humans

Infants acquire ureaplasmas during passage through the birth canal (Foy et al 1970). Foy et al (1970) found that when the genital tract was sampled within 2 days of birth, 9/42 (21%) females and 1/52 (2%) males had cultivable ureaplasmas. Klein et al (1971) reported that if the membranes were unruptured and delivery was by Caesarean section then none of the infants tested (0/20) had cultivable ureaplasmas at birth. In addition to the genitals, ureaplasmas colonized the umbilicus, nose, throat and, less often, the conjunctiva (Klein et al 1969, Foy et al 1970, Prentice et al 1977, Rudd & Cassell 1984). The ureaplasmas acquired at birth were rapidly lost (Foy et al 1970, Hammerschlag et al 1978). Ureaplasmas have rarely been isolated from boys or girls between the ages of 2 to 13 years old (Csonka et al 1966, Foy et al 1975). The incidence of isolation of ureaplasmas from girls between the ages of 13 to 18 years old ranged from 0/44 (Csonka et al 1966) to 31/296 (10%) (Foy et al 1975). It is generally accepted that ureaplasmas are seldom isolated prior to the

onset of sexual activity (McCormack et al 1972, Taylor-Robinson & McCormack 1979). Thereafter, in adults, as the number of consorts increases so does the incidence of ureaplasmas in the genital tract. McCormack et al (1973) found that 39/87 (45%) of sexually active men (having three or more sexual partners) and 9/12 (75%) of sexually active women (McCormack et al 1972) were colonized by ureaplasmas. Holmes et al (1985) have suggested that almost all sexually active women harbour ureaplasmas in their vaginas. These data and also those for colonization of children indicate that females are more readily colonized than males (Taylor-Robinson & McCormack 1979). Ureaplasmas colonize the anal canal of women (Munday et al 1981) which would facilitate re-infection of the genital tract and may contribute to the higher colonization observed in females. In the absence of antimicrobial therapy, ureaplasma colonization of the adult genito-urinary tract is persistent (Holmes et al 1974, MacLeod et al 1976, Sautter & Brown 1980) yet little is known about the attachment of ureaplasmas to eukaryotic cells or how they remain localized.

Ureaplasmas have been associated with a variety of diseases of humans (Table 2). In many of these diseases (Table 2) the causal role of ureaplasmas is often questioned. Traditionally, microorganisms are thought to be pathogenic if the relationship between a microbe and a disease fulfills Koch's postulates; however, this is not an absolute requirement. Certain established pathogens do not fulfill these postulates since the isolation of the

Table 2 Association of *Ureaplasma urealyticum* with diseases of humans.

Disease	Evidence	Comments
Urethritis (NGU)	+	Causes some cases (Shepard 1974, Taylor-Robinson *et al* 1977a, Bowie *et al* 1977, Taylor-Robinson 1984); estimated to cause 30% to 40% of NGU (McCutchan 1984).
Arthritis	+	Based on case studies of hypogammaglobulinaemic patients. (Stuckey *et al* 1978, Webster *et al* 1978, Waites *et al* 1984)
Prostatitis	±	Associated with a few cases of chronic disease (Weidner *et al* 1978, Weidner *et al* 1980, Brunner *et al* 1983) but causal role not proven.
Abortion	±	Maternal and fetal infections associated with spontaneous abortions (Discroll *et al* 1969, Stray-Pederson *et al* 1978, Quinn *et al* 1983) but causal role not proven.
Infertility	±	Associated with disease (Kundsin *et al* 1967, Horne *et al* 1973, Cassell *et al* 1983, Upadhayaya *et al* 1983) but role questioned by Gump *et al* (1984).
Chorioamnionitis	±	Associated with disease (Caspi *et al* 1972, Shurin *et al* 1975, Kundsin *et al* 1984) but causal role not proven.
Low birth weight	±	Associated with low birth weight infants (Klein *et al* 1969, Eldner *et al* 1971, Kundsin *et al* 1984) but causal role not proven.
Respiratory	±	Associated with few cases (Quinn *et al* 1983, Quinn *et al* 1984) but causal role not proven.

+ = good; ± = poor or controversial

Modified from Taylor-Robinson and McCormack (1980a), and Taylor-Robinson (1984) by exclusion of conditions where no evidence exists for ureaplasma involvement.

microorganism does not necessarily indicate disease (e.g. Staphylococcus aureus does not cause skin lesions in normal persons unless a foreign body, such as a suture, is also present (Morse 1980) and Clostridium difficile in the intestine does not always indicate enterocolitis (Swartz 1980)). The relationship between ureaplasmas and the sexually transmitted disease (STD) nongonococcal urethritis (NGU) fulfills Koch's postulates (Taylor-Robinson et al 1977, Taylor-Robinson 1980a, Taylor-Robinson 1980b, McCutchan 1984, Taylor-Robinson 1984). NGU will be discussed in some detail since it is the disease caused by U. urealyticum that has been most extensively studied. The most controversial issue with respect to pathogenicity of ureaplasmas and their role in NGU is the presence of these organisms in a large proportion of asymptomatic males (Bowie et al 1976). Shepard's (1974) quantitative study of ureaplasmas in men with NGU who received suboptimal tetracycline therapy showed a strong correlation between the number of detectable ureaplasmas and the clinical course of the disease. Shepard's (1974) study provided the first quantitative data supporting the role of ureaplasmas in NGU. Taylor-Robinson et al (1977) demonstrated conclusively the ability of ureaplasmas to cause disease. They inoculated themselves with a strain of serotype 5 ureaplasma isolated from a patient with NGU and monitored the NGU that subsequently developed. Bowie et al (1977a) showed the importance of matching patients for sexual activity and of considering chlamydia-negative patients. When the patients

in this latter study were matched for three or fewer sexual partners and were chlamydia-negative, ureaplasmas were clearly implicated as the cause of NGU. The persistence of NGU after tetracycline therapy and the isolation of tetracycline resistant ureaplasmas from males who were negative for chlamydia (Ford & Smith 1974, Stimson 1981) supported the demonstration by Taylor-Robinson et al (1977) of the role of ureaplasmas in the etiology of NGU.

Gonorrhea is the most commonly reported communicable disease in both Canada (Canadian Diseases Weekly Report 1984) and the United States of America (Morbidity Mortality Weekly Report 1983). Although gonococcal urethritis (GU) is a reportable disease in the United States of America and Canada, NGU is not. The relative incidence and the etiologies of infectious urethritis as estimated by Mellors (1983) are shown in Table 3. In England and Wales, both GU and NGU are reportable diseases and comparisons can be made. Willcox (1972) showed that in 1951 the incidence of NGU was slightly less than that of GU (approximately 11,000 cases compared to 15,000 cases). By 1965, NGU was reported more frequently than GU (29,000 cases and 28,000 cases respectively) (Willcox 1972). Since that time there has been a steady increase in the incidence of NGU compared to that of GU; by 1981 there were three times as many cases of NGU reported than of GU (approximately 90,000 cases compared to 30,000 cases reported in 1981) (Chief Medical Officer, 1984). McCutchan (1984) pointed out that the incidence of

Table 3 Infectious etiologies of male urethritis

	Infectious Urethritis (%)
I. Gonococcal Urethritis (GU)	30-50
II. Nongonococcal Urethritis (NGU)	50-70
	% NGU
Chlamydia trachomatis	40-50
Ureaplasma urealyticum	20-30
Unknown agent(s) (tetracycline-suppressible)*	20-25
Trichomonas vaginalis	5
Herpes simplex	rare
Candida albicans	rare
Endourethral syphilitic chancre	rare
Condylomata accuminata	rare

Excerpt from Mellors (1983).

* It has been suggested that Mycoplasma genitalium (Tully et al 1981) and an anaerobic bacillus (Fontaine et al 1982) may be two such agents.

NGU in England and Wales was subject to diagnostic and reporting biases. McCutchan (1984) speculated that over the past decade the incidence of NGU compared to that of GU has been a stable ratio of approximately two. This information indicates that NGU is of great importance in human health yet very little is known about U. urealyticum which is one of the etiologic agents of NGU.

Because effective treatment for GU (usually penicillin or its derivatives) is ineffective against the agents of NGU, for which tetracycline is the drug of choice (Holmes et al 1967, Willcox 1972, Mellors 1983), this predominance of NGU over GU has critical implications for patient therapy. The clinical features of symptomatic NGU compared to those of GU are shown in Table 4. The diagnosis of NGU is made by establishing the presence of urethritis and the absence of gonococcal infection (Mellors 1983). The presence of five or more polymorphonuclear leukocytes (PMN) per high power field on microscopic examination of an urethral exudate is considered confirmatory of urethritis (Swartz et al 1978, Bowie 1978, McCutchan 1984). The absence of Gram-negative intracellular diplococci on direct microscopy of urethral exudate rules out gonorrhoea with 95% to 99% reliability (Jacobs & Krauss 1975, Mellors 1983). Microscopy is most commonly used for determining whether a patient should be treated for GU or NGU. Arnold and Kleris (1980) recommended that "borderline" smears (few PMN, or PMN with extracellular diplococci) of men with urethritis should be treated as for

Table 4 Clinical characteristics of gonococcal and nongonococcal urethritis

Clinical Characteristics	Gonococcal Urethritis	Non-Gonococcal Urethritis
Incubation period	2-8 days	10-20 days
Onset	Abrupt	Gradual
Dysuria	Prominent (occasionally absent)	Variable (often absent)
Urethral discharge	(Percent of 41)	(Percent of 77)
purulent	73%	36%
white	27%	55%
clear	0	9%

Excerpt from Handsfield and Bowie (1979).

NGU. Between one-third to one-half of the men with GU who were treated with a penicillin subsequently developed post-gonococcal urethritis (PGU) which was indistinguishable from NGU (Csonka et al 1966, Oriel et al 1975, Kinghorn 1978, Terho 1978, Jahn et al 1983, Csango et al 1984). PGU is usually due to an initially mixed infection of N. gonorrhoeae and an NGU agent (Csonka et al 1966, Oriel et al 1975, Willcox 1979, Jahn et al 1983, McCutchan 1984). Once the symptoms of gonorrhea are erradicated by treatment with a penicillin the NGU, which has a longer incubation period (Table 4), becomes apparent. Csonka et al (1966) reported that 25/63 (40%) of men with GU also had U. urealyticum and 5/50 (10%) had M. hominis. Oriel et al (1975) reported that 11/44 (25%) of men with GU also had C. trachomatis. Jahn et al (1982) reported that of 143 males with gonorrhea, C. trachomatis was concurrently present in 41 (29%), U. urealyticum in 48 (34%), and M. hominis in 19 (13%), supporting previously reported isolation rates. Although M. hominis has been shown not to be an etiological agent of urethritis in males (Mellors 1983), both U. urealyticum and C. trachomatis can cause NGU (Table 3). Several reports have recommended that physicians be certain they have chosen adequate therapy because patients may have multiple sexually transmitted diseases (Terho 1978, Arya 1981, Fiumara & Calhoun 1982, Csango et al 1984). Not only do these microorganisms co-exist in the host but they can be co-isolated in vitro if the growth medium is suitable. The

co-isolation of genital mycoplasmas with N. gonorrhoeae has been given little consideration.

3. Pathogenic features of genital mycoplasmas

The ability of U. urealyticum to cause urethritis is poorly understood. To understand the in vivo process, the pathogenic characteristics of U. urealyticum must be determined. In humans the primary location of ureaplasmas is, of course, the urogenital tract. To prevent mechanical expulsion by urine or mucous secretion, ureaplasmas must remain localized. Shepard (1957) first recognized the close association between ureaplasmas and exfoliated urethral epithelial cells. Mazzali and Taylor-Robinson (1971), using ureaplasma strains SP1467 and "Johnson", unsuccessfully attempted to establish a chronic infection of L132, HeLa and Vero cell cultures. Ureaplasmas could no longer be detected 3 to 4 days after inoculation and only minimal cytopathic effects (CPE) were observed. They noted that the addition of 0.05% (w/v) (8.3 mM) urea increased the number of viable ureaplasmas. Masover et al (1976a) reported that infection of WI-38 cells with the type strain T-960 resulted in decreased plating-efficiency and decreased nuclear-labelling of WI-38 cells. However, four days post-infection ureaplasmas were no longer detectable by conventional culture methods. Kotani and McGarrity (1984) reported that ureaplasma growth in cell cultures varied with the ureaplasma strain and the cell line used. Taylor-Robinson (1979) suggested that it was possible, but unlikely, that ammonia production by ureaplasmas could be responsible for

the minimal CPE observed with cell cultures. Only McGarrity (1985) has successfully produced chronic ureaplasma infection of cell cultures. He reported that U. urealyticum strain T-960 remained associated with 3T-6 and HeLa cells for at least four serial passages of the monolayer. This association of ureaplasmas with 3T-6 and Hela cells over about 16 days exceeded the 3 to 5 days duration of infection reported previously (Mazzali & Taylor-Robinson 1971, Masover et al 1976). McGarrity (1985) reported that after association with eukaryotic cells U. urealyticum serotype T-960 would no longer grow in vitro on certain media. This indicates that association with eukaryotic cells somehow modifies the growth requirements of the ureaplasmas.

Ureaplasmas could be detected at least 13 days post-infection in Fallopian tube organ cultures but there was no discernable CPE (Taylor-Robinson & Carney 1974). However, Stalheim et al (1976) reported that infection of bovine oviduct organ cultures with U. urealyticum resulted in loss of ciliary activity and deciliation and desquamation. Stalheim and Gallagher (1977) duplicated the CPE that was produced by U. urealyticum by adding either ammonia or urease to oviduct explants. They suggested that liberation of ammonia by the ureaplasma urease may be responsible for the lesions produced by ureaplasma infection.

Ureaplasmas have been shown to adhere to sperm (Fowlkes et al 1975, Swenson & O'Leary 1980, Busolo & Zanchetta

1984). Busolo and Zanchetta (1984) have reported that in an *in vitro* model, ureaplasmas may mask sperm-membrane recognition sites and impair sperm-penetration of zona-free hamster eggs.

Although cell culture and Fallopian tube organ-explants infected with ureaplasmas showed little CPE, Kraus *et al* (1977) detected histological changes in their mouse subcutaneous chamber model. Inoculation of the subcutaneous chambers with ureaplasmas that were isolated from NGU patients elicited an intense PMN response with giant cells in the mice, followed in four weeks by histiocytes (Kraus *et al* 1977). Ureaplasma strains isolated from humans were reported to cause mastitis when inoculated into goats and mice (Gourlay *et al* 1973, Howard *et al* 1975).

Friedlander and Braude (1974) demonstrated that when killed or viable ureaplasma strains isolated from humans were inoculated into the rat bladder, urinary stones were formed. The hydrolysis of urea apparently induces hyperammoniauria. This results in supersaturation of the urine which is believed to cause the struvite in the urine to crystallize and form urinary stones. Friedlander and Braude (1974) suggested that the ureaplasmas that were bound to epithelial cells in the urinary tract may act as the nidus for localized stone formation. Clerc *et al* (1984), using a similar rat model, confirmed Friedlander and Braude's results and also demonstrated that the urease inhibitor, acetohydroxamic acid, effectively inhibited stone formation. They postulated that the ureaplasma urease

enzyme was a major factor in stone formation. Recently, Grenabo (1985) showed that U. urealyticum could induce crystallization in synthetic urine. The role of ureaplasma or its urease enzyme in kidney stone formation in humans is unproven.

Ureaplasmal infection was established by intraurethral inoculation of chimpanzees with ureaplasmas but no PMN response was observed (Taylor-Robinson et al 1978). The strains used for inoculation had been passaged in vitro. Later studies with nonpassaged ureaplasmas resulted in a PMN response (Taylor-Robinson 1979). These studies suggested that non-passaged ureaplasmas might attach to eukaryotic cells but provided no information about the nature of the receptors.

The primary focus of each of the pathogenicity studies discussed in Sect. 3 was to establish chronic ureaplasma infection. There have been few attempts to characterize the attachment of U. urealyticum to eukaryotic cells and this important process remains poorly understood.

Mårdh (1983) has suggested that M. hominis is frequently a "neglected human pathogen". Although it can be isolated from the male urethra, it has been shown not to be an etiologic agent of NGU (Holmes et al 1975, Bowie et al 1977b). There is, however, convincing evidence that M. hominis is associated with upper urinary tract infections (Mårdh et al 1972, Thomsen & Lindskov 1979, Brunner et al 1983) and with pelvic inflammatory disease (PID) in women

(Mårdh & Westrom 1970, Møller et al 1981, Handsfield 1983). M. hominis has been shown capable of invading the circulatory system (McCormack et al 1975, Taylor-Robinson & McCormack 1980b).

4. Characteristics of Neisseria gonorrhoeae

N. gonorrhoeae is a Gram-negative diplococcus belonging to the Family **Neisseriaceae**. The individual cocci are approximately 0.8 µm x 0.6 µm and after approximately 48 hrs incubation at 37°C in vitro they undergo rapid autolysis which produces bizarre shapes. N. gonorrhoeae and other neisseria oxidize dimethyl- or tetramethyl-p-phenylenediamine. N. gonorrhoeae is distinguished from other neisseria by its ability to ferment glucose but not maltose or sucrose. The colonies of N. gonorrhoeae have been classified T_1 to T_4 (Kellogg et al 1963). The classification depends on the presence (T_1) or absence (T_4) of pili (Swanson et al 1971). Clinical isolates are predominantly piliated (T_1) with successive subculture in vitro gradually producing predominantly non-piliated (T_4) strains (Gotschlich 1980). Competence for transformation is greater in piliated N. gonorrhoeae than in their non-piliated isogenic variants (Sparling 1966, Cannon & Sparling 1984). Pili appear to function as important but not sole effectors of attachment to eukaryotic cells (Swanson 1973, Swanson 1983, Sugasawara et al 1983).

The major outer membrane protein, designated P.I, forms cationic selective pores in the lipid bilayer. Migration of P.I on SDS-PAGE is not altered by pre-treatment at 100°C for

10 min (Greco et al 1980, Blake & Gotschlich 1983, Young et al 1983). Blake and Gotschlich (1983) suggested that insertion of P.I into eukaryotic membranes may be involved in the internalization of gonococci by epithelial cells. Serotyping schemes based on heterogeneity of the P.I component have been proposed (Wang et al 1977, Sandstrom et al 1982a, Sandstrom et al 1982b, Knapp et al 1984). The serotyping schemes are based on sera that react with P.IA or P.IB (Sandstrom et al 1982b, Knapp et al 1984). Sandstrom et al (1984) demonstrated that 85/101 (84%) of isolates from patients with disseminated gonococcal infection belonged to the P.IA group (called serogroup WI by the authors). N. gonorrhoeae strains with low molecular weight P.I's were more serum resistant than strains with P.I's of high molecular weight (Hildebrandt et al 1978, James et al 1982). Transformation studies have shown the locus for P.I is closely linked to the locus for serum resistance (Sparling et al 1978, Cannon & Sparling 1984).

Epidemiological studies have utilized serotype, as described above, and auxotype as markers for studying the geographical distribution and the temporal changes in N. gonorrhoeae populations (Sandstrom et al 1984, Knapp et al 1984). Auxotyping was introduced by Carifo and Catlin in 1973 and is based on differing nutritional requirements of N. gonorrhoeae strains. Auxotype has been correlated with disseminated gonococcal infection and plasmid content. Arginine-hypoxanthine-uracil requiring (AHU^-) auxotypes have

been isolated most frequently from patients with disseminated gonococcal infection (Sandstrom et al 1983, Cannon et al 1983). Brunham et al (1985) have suggested that all strains of N. gonorrhoeae isolated from disseminated gonococcal infections have the P.IA serotype, irrespective of their auxotype. Dillon et al (1981) showed that all of the proline-citrulline-uracil requiring (PCU^-) strains that they tested lacked plasmids. Copley et al (1983) reported that serotype, based on P.I antigens, and auxotype were stable characteristics during natural transmission.

5. Characteristics of HeLa cells

Some of the previous studies on ureaplasma pathogenicity have employed HeLa cells. Since HeLa cells are used in the present study, the history and characteristics of this cell line will be briefly reviewed. This continuous cell line originated from biopsy material taken from the cervical carcinoma of Henrietta Lacks in 1951 (Gey et al 1952). Originally classified as an epidermoid carcinoma, it was later reclassified by Jones et al (1971) as an adenocarcinoma. HeLa cells are epithelial-like. The cell shape and distribution of the many microvilli depend on the stage of the growth cycle (Lundgren & Roos 1976). These cells grow optimally at 36°C. The ATCC recommend growing HeLa 229 cells at pH 7.5, however, Eagle (1973) demonstrated that growth of HeLa cells can occur over a wide pH range (6.4 to 8.0) with an optimal pH of about 6.8. HeLa cells can be grown in defined synthetic medium supplemented with

either serum or a defined hormone-supplement (Hutchings & Sato 1978). Gartler (1968), using isoenzymes as a method of identifying eukaryotic cell cultures, demonstrated that HeLa cells produce glucose-6-phosphate dehydrogenase isoenzyme A, which is a sex-linked isoenzyme found only in blacks and predominately (about 70%) in females. This unique isoenzyme was the basis for his suggestion that many human eukaryotic cell lines had been contaminated with HeLa cells. This observation was later confirmed by the human leukocyte antigen serotyping scheme of Espmark et al (1978). This type of intraspecies contamination was most likely the result of the wide distribution of HeLa cells, their hardy growth characteristics and poor tissue culture technique. HeLa cells have been used as the model system to study the adherence characteristics of a wide range of bacteria. Because they have been extensively characterized, are easy to cultivate, and originated from the human genital tract, HeLa cells were chosen for use in this study of ureaplasma adherence.

6. Objectives of this research

Although U. urealyticum causes urethritis which affects a large portion of adult males, there have been few attempts to characterize the interaction of this microorganism with other prokaryotic or eukaryotic cells. The objectives of this study were to analyze the interactions of ureaplasmas with other cells using Neisseria gonorrhoeae and HeLa 229 cells as the representative models. The genital mycoplasmas, U. urealyticum and M. hominis, have not been

considered in previous studies on the effect of genital tract microorganisms on N. gonorrhoeae. In the present study, co-isolation of genital mycoplasmas and N. gonorrhoeae from urethral specimens was investigated to determine the duration of this association in vitro and if there was any correlation between the ureaplasma serotypes and the neisseria auxotypes of the co-isolates obtained. The interrelated growth of M. hominis with N. gonorrhoeae auxotype standard strains in vitro was examined to determine if such co-existence could effect auxotypes, plasmid profiles or protein banding patterns of the neisseria.

Although the results of previous studies have suggested that ureaplasmas could attach to eukaryotic cells, these studies were limited to recovery of viable ureaplasmas from infected eukaryotic cells (Mazzali & Taylor-Robinson 1971, Taylor-Robinson & Carney 1974) or to monitoring the effect of infection on the metabolism of the eukaryotic cells (Masover et al 1976a). The present qualitative and quantitative analyses examine in greater detail the association and more specifically the attachment of ureaplasmas to HeLa 229 cells. Knowledge of outer membrane components of ureaplasmas will be required to determine which components may be involved in attachment to eukaryotic cells. Preliminary characterization of the U. urealyticum membrane was undertaken to determine which of the outer membrane components produced by the ureaplasmas and which had been adsorbed from the horse-serum in the growth medium.

CHAPTER II. MATERIALS AND METHODS

1. Cultures

1.1 Prokaryotic Cells

Ureaplasma urealyticum strains 27 (serotype 3), and T960-CX8 (serotype 8) as well as *Mycoplasma hominis* strain 14027 (American Type Culture Collection (ATCC)) were provided by Dr. J.A. Robertson (Department of Medical Microbiology, University of Alberta).

Escherichia coli strains designated 481, 369, 77, 433 and 285 which contained plasmids of 2.6, 23, 38, 60 and 96 megadaltons (Mdal), respectively, were provided by Dr. D.E. Taylor (Department of Medical Microbiology, University of Alberta).

The *Neisseria gonorrhoeae* auxotype standard strains:

C_1: ornithine requiring (O^-)

C_2: non-requiring of the supplements tested (N.R.)

C_3: proline-citrulline-uracil requiring (PCU^-)

C_4: proline-ornithine requiring (PO^-)

C_5: citrulline-uracil-hypoxanthine requiring (CUH^-)

C_6: ornithine-uracil-hypoxanthine requiring (OUH^-)

were provided by Dr. J. R. Dillon (Laboratory Centre for Disease Control, Ottawa).

The wild-type strains of *N. gonorrhoeae*, *M. hominis* and *U. urealyticum* were isolated from urethral exudates of men attending the Social Hygiene Services, Sexually Transmitted Diseases Clinic in Edmonton, Alberta. Our study

of urethral exudates from males with gonorrhea was undertaken with the cooperation of Dr. B. Romanowski, Director.

All prokaryotic stock cultures were maintained by storage at -70°C in an appropriate broth medium with 10% (v/v) added sterile glycerol (Sigma Chemical Co., St. Louis, MO).

1.2 Eukaryotic Cells

HeLa 229 (CCL2.1) tissue culture cells were obtained from the ATCC. Stock cultures were maintained by suspending cells in supplemented Minimal Essential Medium (MEMS) (Sect. 2.2) containing 10% (v/v) dimethyl sulfoxide (Sigma Chemical Co.) and storing the suspensions in sterile vials in the gas phase above liquid nitrogen.

2. Media and Solutions

2.1 For Prokaryotic Cells

U. urealyticum was grown on genital mycoplasma (GM) agar or in bromothymol blue (B) broth (Robertson 1978). The composition of these media are given in Appendix I (Sect.1 & 2, respectively). GM agar was used within 48 hrs of preparation. B broth supplemented with 20 µg/ml lincomycin (UpJohn Co. of Canada, Don Mill, Ontario) or with 0.5% (w/v) arginine (Sigma Chemical Co.) was used to isolate U.urealyticum and M. hominis, respectively, from urethral specimens.

E. coli strains were grown on nutrient agar (Difco Laboratories, Detroit, MI).

N. gonorrhoeae was grown on GCS agar that was composed of GC medium base (Difco Laboratories) supplemented with 1% (v/v) IsoVitalex (Becton Dickinson and Co., Cockeyville, MD) and 5% (v/v) mycoplasma-tested, pooled, normal-horse serum (Gibco Laboratories, Grand Island, NY). GCS agar prepared without serum was designated GC agar. Wild-type strains of N. gonorrhoeae were isolated from urethral exudate on Edmonton Provincial Lab Medium (Anand and Kadis, 1980). Subsequent subcultures of these strains were made to chocolate agar (approximately 20 ml volume) that had been supplemented by spreading 0.1 ml IsoVitalex over the surface prior to inoculation. The auxotyping scheme of Hendry and Stewart (1979) was used. The media preparation procedure (Appendix I, Sect. 5) was that of Dillon (1983).

Hepes (H) buffer (Appendix I, Sect. 6) was used for washing and suspending the bacterial strains.

2.2 For Eukaryotic Cells

HeLa cells were grown in supplemented Minimal Essential Medium (MEMS) (Flow Laboratories, McLean, VI) which was prepared as described in Appendix I, Sect. 7. Phosphate buffered saline (PBS) (pH 7.0) or H buffer (pH 6.0) was used to wash the HeLa cells.

Standard agar and standard broth (Appendix I, Sect. 3 & 4, respectively) were used for testing HeLa cultures for mycoplasma contamination.

3. Microscopy

3.1 Light microscopy

An inverted Leitz Dialux 20 microscope (Ernst Leitz

Ltd., Wetzlar, West Germany) at x40 or x100 magnification was used to observe colonies on agar medium by light microscopy. This microscope was equipped with a MPS-51 camera and MPS-45 Photoautomat System (Wild Heerbruug Ltd., Heerbruug, Switzerland).

3.2 Fluorescence microscopy

A Leitz Dialux 20 fluorescence microscope (Ernst Leitz Ltd.) at x400 magnification was used with the following filters:

I_2 (450 to 490 nm) for detection of fluorescein isothiocyanate (FITC),

A (330 to 385 nm) for detection of the Hoechst 33258 stain.

The ultraviolet light source was positioned to provide epi-illumination. This microscope was equipped with a MPS-51 camera and MPS-45 Photoautomat System.

3.3 Transmission electron microscopy

Specimens were fixed, embedded, and stained by the standard procedures given in Appendix II, Sect. 1. A gelatin-overlay technique (Meloni et al 1980) was used to keep the N. gonorrhoeae colonies in place during processing. A Philips 300 transmission electron microscope (Philips, Eindhoven, The Netherlands) at an accelerating voltage of 80 Kv was used for all transmission electron microscopy (TEM) of sectioned material.

3.4 Scanning electron microscopy

The specific protocols for fixation and dehydration are

given in Appendix II, Sect. 2. After dehydration, the samples were further processed using the CO_2 critical point method (Critical point drier, SeeVac Inc., Pittsburgh, PA). The specimens were mounted on aluminum stubs (JBM E.M. Supplies, Montreal, Quebec) with E-Kote No. 3040 silver paint (Acme Chemicals and Insulation Co., New Haven, CT) and then coated with about 60 Å of carbon using a model NRC 3115 carbon coater (Varian, Palo Alto,-CA). The carbon-coated coverslips were observed with a Philips SEM 505 scanning electron microscope (Philips). The accelerating voltage used was 20 Kv or 30 Kv.

4. Protein analysis procedures

4.1 SDS-PAGE of proteins

The sample was suspended in 50 µl of modified Laemmli's sample buffer (Appendix II, Sect. 3.1.3). This suspension was exposed to ultrasound for about 8 secs, using a sonicator water bath (Fisher Scientific Co., Fairlawn, N.J.) and then the suspension was heated at 100°C for 10 mins. The sample (10 µl) was applied to a lane of a polyacrylamide gel.

A Bio-Rad 16-cm slab cell polyacrylamide electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) was used for electrophoretic separation of the proteins in the sample extract. The composition of the SDS-PAGE reagents are given in Appendix II, Sect. 3. The stacking gel was 5% and the running gel was either 12.5% or 14%. The gels were run at 10 watts constant power per gel for 3.5 hrs. The gels were fixed in destaining solution for

approximately 8 hrs before storage in 7.5% (v/v) acetic acid. Alternatively, some of the gels were stained with a silver stain (Appendix II, Sect. 5). For certain ^{125}I analyses, the polyacrylamide gels were dried for 3 hrs using a model 224 Gel Slab Drier (Bio-Rad Laboratories). Autoradiography was carried out at -70°C using Cronex Xtra Life Lightning-plus intensifying screens (DuPont de Nemours and Co., Wilmington, DE) and X-Omat AR-5 film (Eastman Kodak Co. Rochester, NY). Exposure times ranged from 24 hrs to 72 hrs.

4.2 Immunoblotting of proteins

The Bio-Rad Trans-Blot apparatus (Bio-Rad Laboratories) was used at 27 volts for 2 hrs to transfer proteins from a polyacrylamide gel to a 0.45 µm nitrocellulose sheet (Schleicher and Schuell, Keene, NH). The composition of the immunoblotting buffer is given in Appendix II, Sect. 3.1.4. The nitrocellulose was probed using the following modification of the Bio-Rad Immunoblot procedure. (Unless stated otherwise, all steps were carried out at room temperature (RT) with constant agitation of the nitrocellulose sheet.) Immediately after electrophoretic transfer, the remaining protein binding sites on the nitrocellulose sheets were saturated by incubating the sheets with a solution containing 3% (w/v) bovine serum-albumin (BSA). The nitrocellulose sheet was then transferred to the first antibody solution. Anti-*U. urealyticum* strain 27 (serotype 3) serum and anti-*M. hominis*

strain 14027 serum were both used at one-to-five-hundred dilution in the 1% (w/v) BSA solution. The nitrocellulose sheet was incubated overnight at 4°C in the appropriate serum, rinsed briefly with ultra-pure water (MilliQ reagent grade water system, Millipore Corp., Bedford, MA) and then washed twice for 10 min each in 100 ml of Tween-20, tris-buffered saline (TTBS). The nitrocellulose sheet was transferred to the second antibody solution for 1 hr. This second antibody solution consisted of horseradish peroxidase conjugated, goat anti-rabbit IgG (Capell Laboratories, West Chester, PA) which was used at a one-to-one thousand dilution in the 1% (w/v) BSA solution. The nitrocellulose sheet was washed briefly in ultra-pure water, then washed twice for 10 min in TTBS and finally immersed in the horseradish peroxidase (HRP) colour development reagent (Bio-Rad Laboratories). The nitrocellulose sheets were developed for 5 to 10 mins. Colour development was stopped by transferring the nitrocellulose sheet to ultra-pure water for at least 10 mins. The nitrocellulose sheet was photographed while still wet. The amido black protein staining method of Batteiger *et al* (1982) was also used to stain duplicate nitrocellulose sheets which had not been incubated with the BSA solution.

5. Preparation of rabbit immune sera

Rabbit, anti-*U. urealyticum* and rabbit, anti-*M. hominis* sera were provided by Dr. J. A. Robertson. These immune rabbit sera were prepared as described by Robertson and Stemke (1979). Briefly, the following method was used.

Mycoplasmas tenaciously adsorb serum components from the growth medium. Therefore, to prevent anti-horse serum antibody production, the ureaplasmas used for immunization were adapted to growth in B broth that was supplemented with rabbit serum instead of horse serum. Mycoplasma cells from cultures that had reached near-maximum titers were collected by centrifugation. Two litres of culture provided sufficient antigen to immunize one rabbit. The cell suspension of mycoplasmas was adjusted to the desired concentration. The antigen in Freund complete-adjuvant (Difco Laboratories) was used for primary injections in each toe pad and subclavically. The antigen was diluted in pyrogen-free saline for the secondary injections. Female San Juan rabbits between 6 to 8 weeks old were immunized as described by Robertson and Stemke (1979). The antisera were sterilized by Seitz filtration and were stored at 4°C.

6. Preparation of eukaryotic cell culture

6.1 Maintenance of culture

Twice weekly the HeLa cell culture was subcultured. When the monolayer of cells had become confluent the medium was poured off and then the monolayer was rinsed with PBS (pH 7.0). The PBS was removed and then 0.25% (w/v) trypsin (Difco Laboratories) in PBS was added to produce a thin film over the monolayer. The culture was incubated at 36°C for about 5 min or until all of the HeLa cells had detached from the flask. The HeLa cells were dispersed by drawing them up and expelling them from a pipette several times.

MEMS was added and then one-third of the trypsinized monolayer was inoculated into a flask containing fresh medium. Sterile disposable $25cm^3$ or $75cm^3$ tissue culture flasks (Corning Glass Works, Corning, NY) were used. All work involving cell cultures was performed in a Class B laminar flow hood.

The HeLa 229 cell line was examined monthly for possible contamination. For examination for bacterial contaminants, the supernatant medium and the HeLa cell suspension were plated onto blood agar. Standard mycoplasma broth and agar were used for examination for mycoplasmal contaminants. To examine monolayers for the presence of non-cultivatable, prokaryotic contaminants, the Hoechst 33258 dye (Calbiochem-Behring, La Jolla, CA) was used in the fluorescent DNA staining method described by Russell et al (1975). Throughout the experimental work the HeLa cell cultures showed no evidence of any such contamination.

6.2 Preparation of monolayers for attachment studies

For attachment assays HeLa cells were grown as monolayers in sterile glass vials (13 mm diameter). Based on hemacytometer counts from a representative experiment, inoculation of vials with 6×10^4 cells produced confluent monolayers containing 1×10^5 cells after approximately 24 hrs incubation at 36^oC. For all attachment assays the HeLa cells were inspected by LM and only confluent monolayers that had been incubated for approximately 24 hrs were used.

For some attachment experiments, the HeLa cells were killed by fixation for 30 mins at 4^oC in 0.05% (w/v)

glutaraldehyde. The glutaraldehyde was washed off with three washes of H buffer. To ensure that no reactive glutaraldehyde was left, the monolayers were incubated at 36°C for 30 mins in H buffer containing 1% (v/v) normal calf serum. The monolayers were washed with three changes of H buffer. These glutaraldehyde treated monolayers were then used in attachment studies.

7 Examination of U. urealyticum

7.1 Identification

Ureaplasmas on GM agar were identified by their small colony size and a positive urease spot test (Shepard & Howard 1970). Briefly, one drop of urease spot test reagent (1% (w/v) reagent grade urea (Fisher Scientific Co.) and 0.8% (w/v) manganese chloride (Sigma Chemical Co.)) was dispensed on colonies growing on GM agar. The culture was incubated at RT for approximately 2 min and then examined by LM. Ureaplasmas degrade the urea resulting in ammonia production. The ammonia reacts with water to produce hydroxide ions and these react with manganese chloride resulting in deposition of manganese dioxide on the surface of the colony. When viewed by LM the colonies appeared a golden-brown colour.

7.2 Colony fluorescence

All ureaplasma strains were serotyped using the colony fluorescence test and the 14-member serotyping scheme of Robertson and Stemke (1982). The following modification of Robertson and Stemke's (1982) colony fluorescence test was

used to detect colonies of ureaplasmas that had grown within colonies of N. gonorrhoeae. H buffer of pH 7.0 with 1% (v/v) calf serum (Flow Laboratories, Inc.) and 0.01% (w/v) sodium azide (Fisher Scientific Co.) was used as the wash buffer. Cultures of neisseria which contained mycoplasmas were grown on GCS agar. Small plugs were cut out of the GCS agar cultures and placed, colony side up, on 0.2 ml of 2% (w/v) agarose (Calbiochem-Behring) in wells of a 96 well microtiter tray (Linbro Scientific Co. Inc., New Haven, CT). A one-in-one hundred dilution of rabbit antisera (200 µl) raised against U. urealyticum strain T960-CX8 was added to each well and the plugs were incubated at 36°C for 15 mins. The antiserum was aspirated and each plug washed with 200 µl of wash buffer four times for 5 mins each. Then 200 µl of a one-in-one hundred dilution of fluorescein isothyiocyanate conjugated (goat) anti-rabbit immunoglobulin (Capell Laboratories Ltd.) (200 µl) was added and the plugs incubated at 36°C for 15 mins. The antiserum was removed and the plugs were washed four times as previously described. The last wash was left on the agar plugs. These were stored at 4°C overnight and then the wash buffer was aspirated and the colonies observed using a fluorescence microscope equipped for epi-illumination (Section 4.2). The fluorescent mycoplasma colonies could be seen within the non-fluorescent colonies of N. gonorrhoeae.

7.3 Preparation of cultures

Robertson (1978) described the colour changes that occur as U. urealyticum grows in B broth (pH 6.0). The B

broth is initially yellow (pH 6.0) and as the ureaplasma culture grows the colour changes to chartreuse and then to green at pH 6.8 and 7.0 respectively. The chartreuse colour that is apparent when the culture reaches pH 6.8 indicates that the ureaplasmas are in the late exponential phase of growth (approximately 10^7 CCU_{50}/ml). The B broth appears green when the medium becomes pH 7.0 and this indicates that the ureaplasmas have attained maximum growth (approximately $10^8 CCU_{50}$/ml). Once maximal growth has been achieved the viability of the ureaplasmas in the culture rapidly declines. For routine propagation, serial one-in-ten dilutions of U. urealyticum in B broth (0.9 ml) were used. Chartreuse-coloured cultures were used as inocula for all experiments to ensure that the ureaplasmas were actively proliferating. When large numbers of ureaplasmas were required, U. urealyticum was grown in B broth until the pH was 6.8 to 6.9 (late exponential stage) and the ureaplasma cells were sedimented at 18,100 x gravity for 20 mins at 4°C using a Type 19 rotor in a Model L5-50 Ultra-centrifuge (Beckman Instruments Inc., Palo Alta, CA). Routinely, the ureaplasma cells obtained from 1 L cultures were resuspended in 1 ml of H buffer.

For some of the attachment experiments, exposure to ultra-violet light was utilized to kill ureaplasmas. Ureaplasma cells (5.8×10^7 CFU) suspended in 1.0 ml of H buffer were dispensed into 60 mm sterile petri dishes (Becton Dickinson & Co.). These cultures were positioned 1

cm from a model UVSL-25 short-wave ultra-violet light (wavelength 254 nm) (Ultra-violet Products Inc., San Gabriel, CA). The shortest exposure time that killed all of the ureaplasmas was 10 mins.

7.4 Enumeration by the CCU_{50} method

The CCU_{50} method, a most probable number method (Reed and Muench 1938, Davis et al 1980), is based upon growth in broth medium. The following CCU_{50} method of Stemke and Robertson (1982) was used for enumerating ureaplasmas. The one-in-ten dilutions were made in vials containing 1.8 ml B broth and the contents of each vial were divided into 225 ul aliquots in 8 wells of a microtiter tray that had been sterilized by U.V. irradiation. When the one-in-ten dilutions had been dispensed, the microtiter tray was sealed with adhesive tape and each well was vented. The tray was incubated until one day had passed without a change in the endpoint; this usually required 4 days. The number of wells in the highest dilution that turned green was used to calculate the endpoint.

7.5 Iodination

Ureaplasmas were labelled with ^{125}Iodine (^{125}I) using the method of Fraker et al (1978). Carrier-free ^{125}I was obtained from Edmonton Radiopharmaceutical Co. (Edmonton, Alberta). Briefly, 1',3,4,6-tetrachloro-3 ,6 ,-diphenyl glycouril (Iodo-Gen) (Pierce Chemical Co., Rockford, IL) was dissolved in chloroform (2 mg/ml) and then 40 µl was dispensed into a 12 x 75 mm acid-washed glass tube (Fisher Scientific Co.). The tube was constantly agitated and the

chloroform was evaporated under a gentle stream of air, leaving a thin film of Iodo-gen on the bottom 5 to 10 mm of the tube. Harvested ureaplasmas (Sect. 7.3) suspended in 0.5 to 0.9 ml of H buffer were pipetted into the Iodo-Gen coated tube. All subsequent manipulations involving ^{125}I were performed in a fume hood. Twenty megabequerels of ^{125}I were added to the ureaplasma suspension. After 5 mins at RT the ureaplasmas were pipetted into a sterile 2.5 ml polypropylene centrifuge tube (Fisher Scientific Co.). To remove unbound ^{125}I, the ureaplasmas were suspended in H buffer and centrifuged at 15,000 rpm for 5 mins in a Microfuge (Geratebau Netherler + Hinz GmbH, Hamburg, West Germany). The ureaplasma pellet was washed three times in this manner and then was suspended in H buffer (1 ml). At least 90% of the protein in a 10 µl sample of the iodinated suspension was precipitated by 10% trichloracetic acid (Fisher Scientific Co.). A model 1270 Rackgamma II gamma counter (LKB, Bromma, Sweden) was used for determining the CPM in samples containing ^{125}I.

7.6 Detection of carbohydrate-like material

7.6.1 SDS-PAGE

A whole cell suspension of harvested ureaplasmas was prepared in 50 µl of modified Laemmli's sample buffer. The sample was boiled for 10 min and then 10 µl of proteinase K (Boehringer Mannheim, Dorval, Que.) (1 mg/ml) was added. The sample was incubated for 2 hr at 60 °C with agitation at 30 min intervals. The sample (10 µl) was added to a lane of

a 14% polyacrylamide gel. After electrophoresis using standardized SDS-PAGE conditions (Sect. 4.1), the gel was reacted with a silver stain (Appendix 2, Sect. 5).

7.6.2 Gas chromatography

The procedure used for gas chramotography was that of Bryn and Jantzen (1982). The harvested ureaplasmas were processed as described in Sect. 7.6.1. A control sample containing only the Laemmli sample buffer was processed in the same manner as the ureaplasma whole cell extract. The proteinase K digests were then dialyzed against 1 L of distilled water. The distilled water was changed every day over a total dialysis time of 4 days. The material from both samples was lyophilized and then used for gas chromatography. About 1 mg of each of the lyophilized samples were transferred to screw-cap septum vials (1.5 ml) (Pierce Chemical Co., Rockford, Ill.). The samples were dissolved in 1 ml of 2 N HCl in anhydrous methanol. The sealed vials were placed in a heating block at 85°C for 16 hrs. The samples were cooled to ambient temperature and transferred to 1.5 ml reaction vials (Pierce Chemical Co.) and then dried by a stream of nitrogen gas. The samples were resuspended in 50 µl of a solution containing equal amounts of trifluoracetic anhydride and acetonitrile (Aldrich Chemical Co., Milwaukee, Wis. and Caledon Labs., Georgetown, Ontario, respectively). The vials were sealed and heated at 65°C for 1 hr. The samples were cooled and then 1 µl volumes were injected into the gas chromatograph. Carbohydrate and fatty acid standards were from Sigma

Chemical Co.. The components in the ureaplasma sample were identified by comparing the retention times of the unknown peaks to the retention times of the standards and the sample buffer control. Each monosaccharide has various ring forms and as a result produces several peaks on the chromatogram (Bryn & Jantzen 1982).

Analyses were carried out on a Varian Vista 600 (Varian Assoc., Sunnyvale, CA) gas chromatograph fitted with a flame-ionization detector and a fused silica capillary column that was 30 m long and had an internal diameter of 0.237 mm and a bonded non-polar liquid phase of 0.25 µm (a DB-1 column from J and W Scientific Co., Rancho Cordova, CA). Helium was used as the carrier gas with a linear velocity of 32 cm per sec. The column temperature was held isothermally at 90°C for 4 min after injection and then programmed to increase to 260°C at a rate of 8°C per min.

7.7 Attachment to N. gonorrhoeae

^{125}I-U. urealyticum strain 27 (Sect. 7.5) was mixed with N. gonorrhoeae strain C_3 in H buffer, and then incubated at 4°C for 1 hr. These microorganisms were spun at 2,000 rpm for 5 min in a benchtop centrifuge. Cells of N. gonorrhoeae form a pellet at such low centrifuge speeds, while ureaplasmas do not. The supernatant fluid was removed and the pellet washed in H buffer. The centrifugation and wash process was performed a total of six times. A control suspension containing only ^{125}I-U. urealyticum was treated similarly. The amount of radioactivity in the supernatant

fluid and cell pellet was measured using a Rackgamma II gamma counter.

7.8 Attachment to HeLa cells

The ureaplasma strains used in the attachment experiments were grown in B broth until the pH was 6.8. The suspension of ureaplasmas was sonicated as described by Robertson (1978). The desired concentration of ureaplasmas was inoculated onto replicate HeLa cell monolayers containing approximately 10^5 cells (Sect. 6.2). For experiments of 24 hrs or longer, the ureaplasmas were suspended in MEMS-amp (pH 6.8) to the desired concentration and then 1 ml was dispensed onto the HeLa monolayer. The vials were stoppered and incubated at 36°C. For experiments of up to 6 hrs duration, the harvested ureaplasmas were suspended in H buffer and then 0.2 ml was dispensed onto each of the monolayers. The vials were stoppered and incubated at 4°C. Normally, incubation was for 1 hr but, occasionally, incubation times of up to 6 hrs were utilized.

7.8.1 Immunofluorescence (IF) assay

Prior to fluorescence staining, the supernatant fluid was removed from the HeLa cell culture and the monolayer was washed three times with 200 µl of H buffer. The monolayer was fixed for 10 mins with acetone and then was rinsed with the wash buffer (Sect. 7.2). The monolayers were then stained as described for colony fluorescence (Sect. 7.2). After "staining", the coverslips were inverted on a drop of mounting fluid (H buffer with 10% (v/v) glycerol) on a glass slide and viewed as described for FM (Sect. 3.2).

7.8.2 Colony forming unit (CFU) assay

Ureaplasma infected monolayers were incubated for an appropriate length of time and then the supernatant fluid was aspirated and transfered to a separate vial. The HeLa monolayer was washed three times with 1 ml of H buffer. The fluid from each wash was aspirated and stored in a separate sterile vial. Using a sterile teflon policeman (Thomas Scientific, Philadelphia, PA), the monolayer was scraped off of the vial into 0.5 ml of H buffer. The cell suspension, supernatant fluid, and washes were sonicated separately and the number of ureaplasmas determined by the colony forming unit (CFU) method on GM agar (Robertson 1978). Briefly, the ureaplasma suspensions were exposed to ultrasound (Robertson 1978) and then used to prepare serial one-in-ten dilutions in B broth. The Miles and Misra (1938) method was used to determine the number of CFU. Four aliquots of 25 µl each were inoculated onto the surface of a GM agar plate. The drops were allowed to dry, then the plates were incubated. An inverted light microscope (Sect. 3.1) and a hand counter were used to count quandrants having 30 to 300 colonies.

7.8.3 ^{125}I-radiolabel (IR) assay

HeLa monolayers grown in vials having a diameter of 13 mm were also used for the ^{125}I-radiolabel (IR) assay. Between 200,000 to 500,000 CPM of ^{125}I-ureaplasmas in 0.2 ml of H buffer were added per monolayer. This represented approximately 10^5 CCU of ureaplasmas added per monolayer. The reaction time was usually 1 hr at 4°C. The supernatant

fluid was removed and the monolayer was washed three times with 0.5 ml of H buffer. The supernatant fluid and the three washes from each monolayer were pooled to determine unbound ^{125}I-ureaplasmas. The HeLa cells were detached by adding 0.2 ml of 0.25% (w/v) trypsin and incubating at 36°C for 10 mins. The trypsinized cells were aspirated into a separate tube. The reaction vial was rinsed with 0.5 ml of H buffer containing 0.1% (v/v) Triton-X 100 (J.T. Baker Chemical Co., Phillipsburg, NJ). This detergent solution was then added to the trypsinized monolayer and the CPM were determined by counting in a Rackgamma II gamma counter. These counts reflected the number of ureaplasmas which had bound to the monolayer.

7.9 Duration of ureaplasma association with HeLa cells

MEMS-amp (Appendix I, Sect. 7) was the medium used for long-term attachment experiments. For some experiments MEMS-amp contained; urea (0.025% w/v) and/or 50 µg/ml gentamicin (MEMS-gent). HeLa cells were grown on 13 mm diameter coverslips held in 60 mm sterile Falcon tissue culture petri dishes (Beckton, Dickinson, and Co.) that were incubated in an incubator containing 7% (v/v) CO_2/balance air. The monolayers were subcultured by scraping off the HeLa cells using a sterile teflon policeman. The HeLa cells were diluted one-to-three with appropriate MEMS medium. One-third of the HeLa suspension was used to inoculate three petri dishes; two of these petri dishes contained sterile 13 mm coverslips. The coverslips were removed at appropriate intervals for IF (Sect. 7.8.1), CCU titre, and

TEM (Sect. 3.3). The culture in the petri dish without coverslips was used for light microscopy and for further passage. The titer of ureaplasmas bound per monolayer was determined by the CCU method. Monolayers on two coverslips were rinsed three times with H buffer. To determine the titer of bound ureaplasmas, the monolayer on one of the coverslips was placed in 0.5 ml H buffer then the HeLa cells were removed by exposure to ultrasound for 30 sec. Light microscropy of the monolayer that had been exposed to ultrasound indicated that no HeLa cells remained attached to the coverslip. Although many of the HeLa cells were disrupted by the sonication, the viability of the ureaplasmas was not altered. Serial one-in-ten dilutions of the HeLa cell suspension were made in B broth. The dilutions were incubated and then the CCU titer was determined. The theoretical limit of detection for the CCU method was 10 ureaplasmas per monolayer. To detect monolayers that had 1 to 10 ureaplasmas, the monolayer on the second coverslip was placed in 10 ml of B broth and incubated.

8. Examination of Mycoplasma hominis

8.1 Identification

Large colony mycoplasmas were identified by colonial morphology, arginine degradation and growth inhibition by species-specific antiserum (Clyde 1964) or by colony fluorescence using species-specific antiserum (Stemke & Robertson 1981). Colonies of M. hominis growing within

colonies of N. gonorrhoeae were detected by the same colony fluorescence method described for U. urealyticum (Sect. 7.2) except that rabbit anti-M. hominis strain 14027 serum was utilized.

9. Examination of Neisseria gonorrhoeae

9.1 Identification

N. gonorrhoeae was identified on the basis of colonial morphology, oxidase reaction, gram-stain reaction, cellular morphology and carbohydrate utilization pattern. The purity of the isolates from urethral exudate was confirmed by three successive transfers of a single, isolated colony onto GCS agar. At each transfer the absence of mycoplasmas was verified by both LM and culture in B broth.

9.2 Auxotyping

Cultures of N. gonorrhoeae were inoculated onto master plates of GCS agar. After approximately 24 hrs incubation, the master plate, in conjunction with a Steer's replicator, was used to inoculate a complete set of auxotyping media (Appendix I, Sect. 5). These media were used to test for the following requirements: proline, citrulline, ornithine, methionine, uracil and hypoxanthine. The N. gonorrhoeae auxotype standard strains (Sect. 1.1) were included as controls for both the procedure and the media. The inoculated auxotyping media were observed at 18 hrs and again at 48 hrs. All of the N. gonorrhoeae strains should grow on the POS medium (the positive control) and none of the isolates should grow on the NEG medium (the negative control) because all N. gonorrhoeae require cysteine and

cystine for growth (Carifo & Catlin 1973). An isolate that fails to grow on the medium lacking proline is deficient in proline synthesis and its auxotype will be listed at P^-. After 48 hrs incubation, growth consisting of fewer than nine colonies or thin films of growth were recorded as negative (Dillon 1983).

9.3 Plasmid extraction

Plasmid DNA was extracted by the screening method of Birnboim and Doly (1979). The DNA extracts were subjected to electrophoresis at 50 v for 9 hrs in Tris-acetate buffer pH 7.8 (Birnboim & Doly, 1979) on a 0.8% agarose (Calbiochem-Behring Corp.) gel in a HE905 Horizontal electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, CA). The gels were stained with ethidium bromide (Sigma Chemical Co.) (1 µg/ml in distilled water) for 1 hr and then washed overnight in distilled water prior to being photographed. The plasmid standards that were isolated from the E. coli strains described in Sect. 1.1 were included on each agarose gel as molecular weight markers.

10. Study of urethral exudate

A total of 102 male patients attending the Social Hygiene Services Sexually Transmitted Diseases Clinic in Edmonton, Alberta from January to March, 1982 were tested. Urethral exudate was collected using a sterile bacteriological loop. From those patients for which the smear of the exudate contained many PMN as well as intracellular diplococci, a urethral swab was taken for

cultures of N. gonorrhoeae and for genital mycoplasmas. This swab was used to inoculate Edmonton Provincial Laboratory Medium (Anand & Kadis 1980) and then the cotton tip of the swab was cut off into a tube of B broth. The B broth was mixed vigorously and 0.1 ml was inoculated into B broth with lincomycin and B broth with arginine for the selective growth of U. urealyticum and M. hominis, respectively. The broth cultures were observed daily; those changing colour were subcultured onto GM agar. Twelve areas of gonococcal growth on the Edmonton Provincial Laboratory Medium plates, usually separate colonies, were picked randomly using separate, sterile wooden applicator sticks and these inocula were transferred to chocolate agar. Small volumes (0.2 ml) of B broth in the wells of sterile microtiter trays were also inoculated to determine which of the sampled areas contained mycoplasmas. This subculturing process of growth on the chocolate agar plates was performed a total of five times. Growth from the original Edmonton Provincial Laboratory Medium, which had been suspended in 1 ml trypticase soy broth (without antibiotics) containing 10% (v/v) glycerol and stored at -70°C, served as the stock culture. For specimens which produced a colour change in B broth, a loopful of this stock suspension was inoculated onto GCS agar for microscopy.

Pure N. gonorrhoeae and U. urealyticum isolates were studied to determine if interrelated growth of these organisms altered the auxotype of N. gonorrhoeae. To establish pure cultures of N. gonorrhoeae, separate colonies

were subcultured to GCS agar as well as B broth. Those colonies of N. gonorrhoeae that did not contain ureaplasmas produced no colour change in the B broth. Single colonies of the ureaplasma-free N. gonorrhoeae were subcultured three times on GCS agar as well as B broth to ensure that the isolates were ureaplasma-free. Pure cultures of ureaplasmas were obtained by subculturing each ureaplasma isolate three times in B broth. The isolate was then subcultured twice in B broth without antibiotics and onto GCS agar without antibiotics to ensure that no neisseria were present.

CHAPTER III. INTERACTIONS OF *UREAPLASMA UREALYTICUM* AND *MYCOPLASMA HOMINIS* WITH *NEISSERIA GONORRHOEAE*

1. Introduction

Despite the widespread knowledge that genital mycoplasmas (*U. urealyticum* and *M. hominis*) and *N. gonorrhoeae* exist together *in vivo*, little attention has been given to their co-isolation and interrelated growth *in vitro*. The aims of this research were to establish the frequency of co-isolation of genital mycoplasmas and *N. gonorrhoeae* from urethral exudate, the duration of their co-existence and to investigate whether *N. gonorrhoeae* characteristics were altered by such co-existence.

2. Co-isolation of genital mycoplasmas and *N. gonorrhoeae* from urethral exudate.

Urethral exudates from 102 men attending a clinic for sexually transmitted diseases were cultured (Ch. II, Sect. 10) to determine the incidence of co-isolation of genital mycoplasmas with *N. gonorrhoeae* (Table 5). To ensure that no mycoplasmas were overlooked, all specimens of urethral exudate were cultured twice. On both occasions, each specimen was cultured in B broth; on the second occasion a culture on GM agar was also included. The colony fluorescence test (Ch. II, Sect. 7.2) was used for screening agar cultures of *N. gonorrhoeae* for *M. hominis* and *U. urealyticum* since FM is more specific than microscopy alone. Thirty-four specimens contained both mycoplasmas and

Table 5 Incidence of *Neisseria gonorrhoeae* and genital mycoplasmas in urethral exudate from men with urethritis.

Isolated microorganisms	Total no.	(%)
N. gonorrhoeae only	68	(67)
N. gonorrhoeae, *U. urealyticum*	27	(26)
N. gonorrhoeae, *U. urealyticum*, *M. hominis*	4	(4)
N. gonorrhoeae, *M. hominis*	3	(3)
Total no.	102	(100)

N. gonorrhoeae (Table 5). The co-isolation rate of U. urealyticum and N. gonorrhoeae was about four-times that of M. hominis and N. gonorrhoeae (Table 5). Twelve colonies from the direct cultures from each of the 102 specimens were chosen randomly and passaged separately for five passages. Of the 102 specimens 20 had at least 1 colony that contained mycoplasmas. Even after these had been passaged 5 times, 9 specimens (45%) still contained mycoplasmas. Further transfers were not performed. By selectively subculturing those neisseria colonies containing mycoplasmas, the co-existence of these microorganisms could be maintained for at least 20 passages (further subcultures were not performed). The species of mycoplasma present did not appear to influence the duration of co-transfer.

In addition to their usual colonial morphologies both M. hominis (Fig. 1A) and U. urealyticum (Figs. 2A, & B) produced "comet"-shaped growth within colonies of N. gonorrhoeae (Fig. 1B, 2C, D, & E). The "head" of the comet probably represents initial growth of the colony, while the "tail" is made up of cells pulled outwardly by the enlarging colony of N. gonorrhoeae. Both M. hominis (Fig. 1A) and U. urealyticum (Fig. 2A,B) were able to grow independently on GCS agar. Detection of mycoplasma colonies was easier after 2 days incubation when colony size was maximum. Regardless of whether colonies of M. hominis grew independently on the GCS agar or within a neisseria colony, they were easily observed by LM (Fig. 1). U. urealyticum,

Figure 1 Interrelated growth of *Mycoplasma hominis* and *Neisseria gonorrhoeae* isolated from an urethral exudate.

The cultures on GCS agar were incubated for 48 hrs at 36°C in 7% CO_2/balance air. Large colony mycoplasmas, in all instances *M. hominis*, growing within and without *N. gonorrhoeae* colonies (N) are indicated by the arrows. The regularly- shaped *M. hominis* colonies are shown in (A), while the comet-shaped pattern of growth of *M. hominis* is shown in (B). The magnification is x50. The bar represents 500 µm.

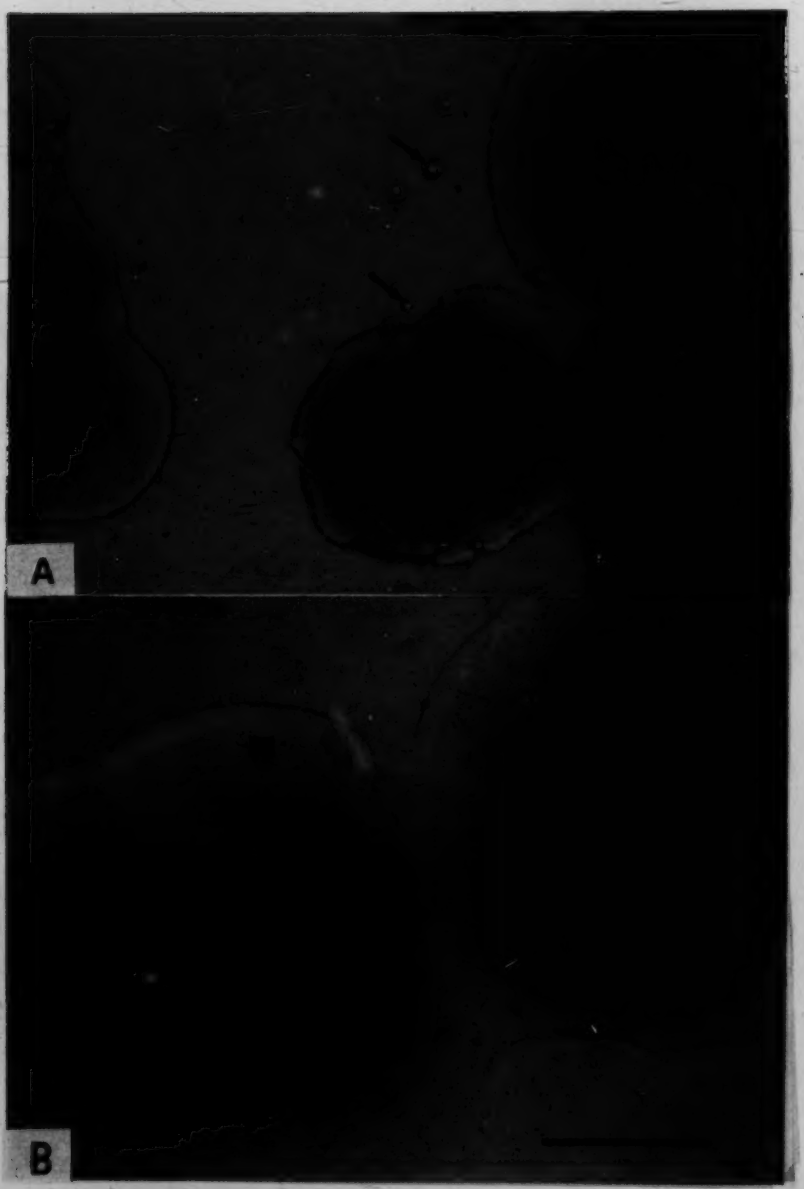
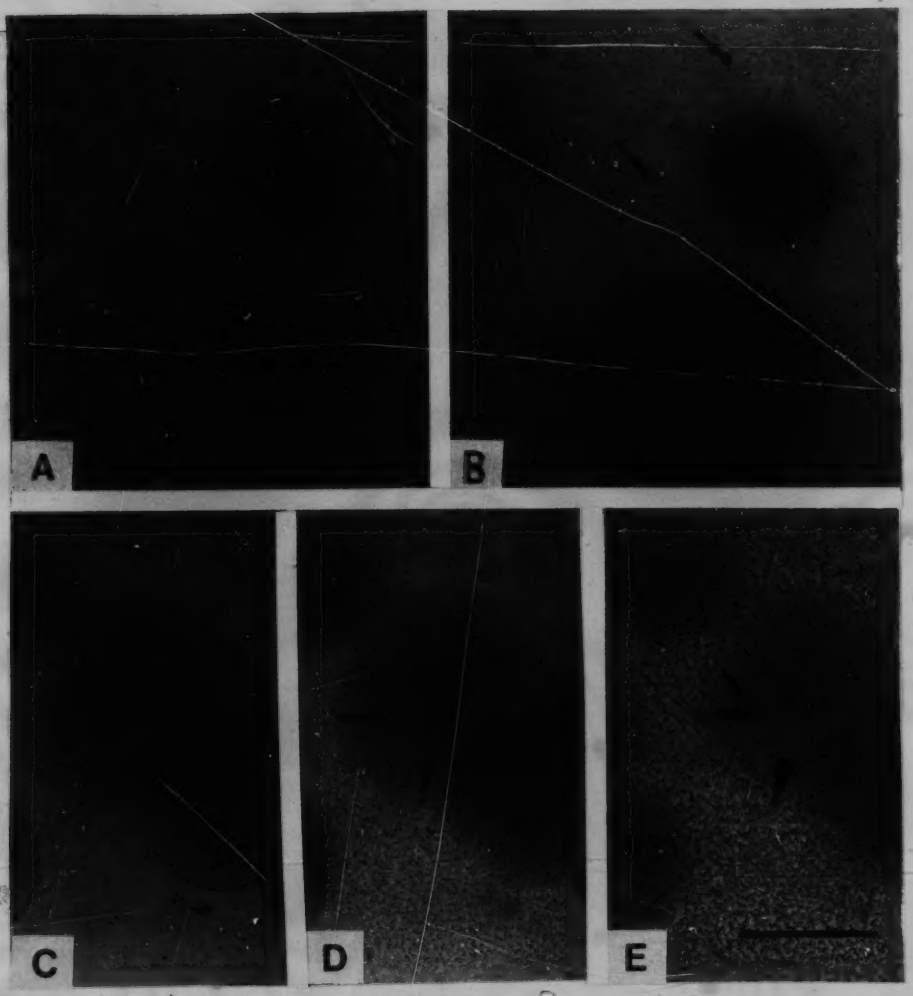

A
B

Figure 2 Interrelated growth of Ureaplasma urealyticum and Neisseria gonorrhoeae isolated from an urethral exudate.

The cultures on GCS agar were incubated for 48 hrs at 36°C in 7% CO_2/balance air. Individual colonies of ureaplasmas are shown in (A) before the urease spot test and in (B) after the spot test. The remaining micrographs show ureaplasma colonies growing within a N. gonorrhoeae colony before the urease spot test (C) and the same colonies 10 secs (D) and 2 min (E) after the spot test reagent was applied. The comet-shaped pattern of ureaplasma growth is now obvious. The magnification is x41. The bar represents 500 µm.


A
B
C
D
E

however, was usually detected only after the application of the urease spot test; this test caused the almost inapparent ureaplasma colonies to turn rapidly to dark brown (Fig. 2).

Nineteen of the pairs of co-isolated neisseria and ureaplasma strains were examined to determine whether such interrelated growth could alter the auxotype of the neisseria strains. Pure cultures of N. gonorrhoeae and U. urealyticum were established by the subculture procedure described in Ch. II, Sect. 10. The N. gonorrhoeae strains were auxotyped (Ch. II, Sect. 9.2) and the U. urealyticum isolates were serotyped (Ch. II, Sect. 7.2) (Table 6). Pure cultures of the co-isolated N. gonorrhoeae and U. urealyticum strains were mixed and the now- contaminated neisseria strains were auxotyped. The auxotype of a given strain of N. gonorrhoeae was the same whether it was a pure culture or when it was mixed with ureaplasmas. The auxotype that occurred most frequently was N.R., followed by PCU^- and P^- (Table 6). All of the U. urealyticum isolates had multiple serotype specificities (Table 6). Serovar 2 was the most common, whereas no serovar 6 or 8 was identified (Table 6).

3. Association of Ureaplasma urealyticum with Neisseria gonorrhoeae

The interrelated growth, particularly the comet-shaped formations (Figs. 1 & 2), suggested that the ureaplasmas might be attached to the neisseria cells within those colonies. The interaction of U. urealyticum to

Table 6 Auxotypes of Neisseria gonorrhoeae and serotypes of Ureaplasma urealyticum co-isolated from urethral specimens.

Specimen Number	N. gonorrhoeae Auxotype**	U. urealyticum* Serotype specificities	Culture purity
9	P^-	10,12,13	mixed*
16	PCU^-	2,12	pure
21	PCU^-	3,9,10,14	mixed
22	N.R.	2,4	pure
23	N.R.	2,4,7,10,11,12	mixed
24	N.R.	2,7,9,12,13	mixed
27	N.R.	2,5,13	mixed
30	N.R.	2,10,11,13	mixed
32	N.R.	1,3,5,10,12,14	mixed
36	PCU^-	1,3,4,13,14	mixed
42	N.R.	12,13	mixed
45	N.R.	9,10,13	mixed
46	O^-	3,12,13,14	mixed
71	N.R.	2,4,10,13	mixed
78	N.R.	9,13	mixed
79	P^-	3,10,13,14	mixed
81	N.R.	10,11,13	mixed
101	N.R.	7,13	pure
111	P^-	7,10,11,13	mixed

* U. urealyticum colonies grown on GM agar were used for serotyping. The culture was considered pure if, when stained with a specific antiserum and a fluorescent second antibody, all detectable colonies were fluorescent; if, under the same conditions some colonies were fluorescent and others were not, the culture was considered a mixture of more than one ureaplasma strain.

** Pure cultures of N. gonorrhoeae on GCS agar were used for auxotyping. Requirements were established for: ornithine (O), proline (P), citrulline (C), uracil (U), and hypoxanthine (H). Strains that required none of these compounds were classified as non-requiring (N.R.). (Ch. II, Sect.9.2)

N. gonorrhoeae was examined by mixing strains of the two species in H buffer and then using centrifugation to separate the free ureaplasmas from those that were bound to neisseria (Ch. II, Sect. 7.7). Viable, ^{125}I-labelled U. urealyticum strain 27 cells (Ch. II, Sect. 7.5) (^{125}I-ureaplasmas) were used for these studies. Initially the N. gonorrhoeae concentration was 5.4×10^6 CFU/ml and after six washes the concentration was 9.4×10^6 CFU/ml. The radioactivity detected in the two fractions indicated that after two washes about 70% of the ^{125}I-U. urealyticum cells were associated with the N. gonorrhoeae cells (Table 7). Even after six washes, 22% (62,000 CPM) of the initial radioactivity was detected in the N. gonorrhoeae pellet, whereas 2.1% (6,000 CPM) of the initial radioactivity was detected in the ureaplasma control (Table 7).

4. Effects of Mycoplasma hominis on in vitro studies of N. gonorrhoeae auxotype standard strains

Auxotype standard cultures are utilized to verify proper preparation of auxotyping media and to provide a basis for comparison when determining auxotypes of wild-type isolates. Obviously, if reproducibility is to be attained, the auxotypes of these standards must be stable. When the auxotype standard cultures obtained for this study were typed, three of those supposed to be auxotrophic (strains C_3, C_4, & C_5) appeared prototrophic (Table 8). All of these neisseria cultures were screened by LM, colony fluorescence (Ch. II, Sect. 7.2) and subculture to B broth

Table 7. Association of ^{125}I-*Ureaplasma urealyticum* strain 27 with *Neisseria gonorrhoeae* auxotype standard strain C_3.

Wash No.	Amount of radioactivity* in pellet (%) Mixture:* *U. urealyticum* and *N. gonorrhoeae*	Control:** *U. urealyticum* only	Ratio: mixture/ control
0	100.0	100.0	1:1
2	73.5	3.5	20:1
4	44.4	2.1	21:1
6	22.0	2.1	11:1

* The *N. gonorrhoeae* and *U. urealyticum* mixture, suspended in H buffer (1 ml), consisted of a pure culture of *N. gonorrhoeae* auxotype control strain C_3 (4.9×10^6 CFU) and a pure culture of ^{125}I-*U. urealyticum* stock culture strain 27 (2.8×10^5 CPM, where the specific activity was 1.5 CPM per ureaplasma cell). The mixture was incubated at 4°C for 1 hr. The bound ureaplasmas were then separated from the free ureaplasmas by centrifugation at 2,000 rpm for 5 min (Ch. II, Sect. 7.7).

** The control, consisting of ^{125}I-*U. urealyticum* strain 27 (2.8×10^5 CPM) suspended in H buffer (1ml), was subjected to the same treatment as the mixture.

Table 8 Auxotypes of the Neisseria gonorrhoeae standard strains used in this study.

Auxotype Standard Strain	Expected auxotype*	Actual auxotype**	M. hominis detected	(Strain designation)
C_1	O^-	O^-	-	(C_1)
C_2	N.R.	N.R.	+	(C_2M)
C_3	PCU^-	N.R.	+	(C_3M)
C_4	PO^-	N.R.	+	(C_4M)
C_5	CUH^-	N.R.	+	(C_5M)
C_6	OUH^-	OUH^-	-	(C_6)

Requirements were established for ornithine (O), proline (P), citrulline (C), uracil (U), and hypoxanthine (H). Strains that required none of these compounds were classified as non-requiring (N.R.) (Ch. II, Sect. 9.2).

* The stated auxotype of the culture sent to us.

** The auxotype as determined in this laboratory.

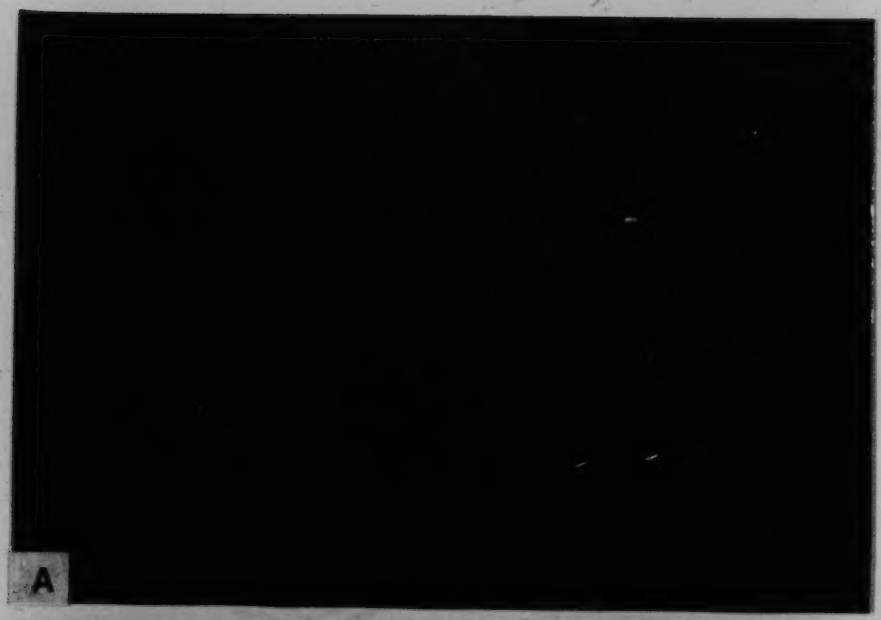
for the presence of mycoplasmas. The cultures of the four prototrophic N. gonorrhoeae, including the three showing improper auxotypes, contained organisms identified by colony fluorescence as M. hominis (Table 8). M. hominis was isolated from most but not all of the multiple frozen stocks of these neisseria strains. Alterations in the auxotype of the N. gonorrhoeae strains were only apparent in those cultures containing M. hominis. The auxotype standard cultures were examined to determine how pure cultures of the N. gonorrhoeae standard strains C_3 and C_5 differed from their mycoplasma-containing counterparts (C_3M and C_5M, respectively) and what effect the removal of M. hominis from the neisseria cultures (C_3M^- and C_5M^-, respectively) had on auxotype, and the plasmid and protein profiles of the latter. The PCU^- and CUH^- auxotypes (strains C_3 and C_5 respectively) were studied to determine if they would return to their original auxotype once the mycoplasmas had been removed. These strains were chosen because the incidence of these auxotypes is higher than the incidence of the PO^- auxotype (Dillon & Pauze 1981). Also, the PCU^- strains do not contain plasmids (Dillon & Pauze 1981). The C_2 strain was not included because auxotype changes would not be expected since it originally was protrophic.

4.1 Microscopy of interrelated growth

Closer visual examination of the neisseria colonies was made by light and electron microscopy. The urease spot-test was negative for both C_3M and C_5M indicating the absence of

Figure 3 Interrelated growth of Neisseria gonorrhoeae auxotype standard strain C_5M and Mycoplasma hominis strain M_5, observed by light and fluorescence microscopy.

The culture on GCS agar was incubated for 48 hrs at 36°C in 7% CO_2/balance air. The light micrograph (A) shows many mycoplasma colonies growing independently and within the neisseria colony. Both regular and comet-shaped growth of the M. hominis colonies are indicated by arrows. The magnification is x30. The bar represents 500 µm. After reaction with rabbit anti-M. hominis serum and then FITC (goat) anti-rabbit immunoglobulin (Ch. II, Sect. 7.2), the comet-like growth fluoresced (B) indicating that the colonies were M. hominis. Arrows denote comet-like structures (B) similar to those seen using light microscopy (A). The magnification is x150. The bar represents 100 µm.


A

B

the more common genital mycoplasma, U. urealyticum. All of the mycoplasma-like colonies visible at x40 magnification fluoresced in the colony fluorescence-test using antiserum to M. hominis. Fluorescence was observed in both the "head" and "tail" components of these comet-shaped colonies (Fig. 3B). The urease spot-test and the colony fluorescence-test were performed on two-day old cultures. The importance of the length of incubation of N. gonorrhoeae containing M. hominis is evident in the transmission electron micrographs of cross-sections of the N. gonorrhoeae strain C_5M (Figs. 4 & 5). The smaller and more electron dense mycoplasma cells are apparent in both 24 hr (Fig. 4) and 48 hr (Fig. 5) cultures of neisseria but are easier to differentiate in the latter since the mycoplasmas have remained intact whereas the neisseria have undergone autolysis.

4.2 Auxotype determination

Photomicrographs of N. gonorrhoeae colonies also showed large numbers of mycoplasmas (Figs. 3, 4, & 5). Metabolic products of the mycoplasmas might provide nutrients for the neisseria. Preliminary experiments were devised to test whether such "cross-feeding" was responsible for the altered auxotypes that were observed (Table 8). The mycoplasma-contaminated strains designated C_3M and C_5M were utilized. Since M. hominis requires sterols, the contaminated neisseria strains were passaged on GC agar which did not contain serum and at each passage the

Figure 4 Transmission electron micrographs of the interrelated growth of a 24 hour old culture of *Mycoplasma hominis* strain M_5 and *Neisseria gonorrhoeae* strain C_5M.

The culture on GCS agar was incubated at 36°C for 24 hrs in 7% CO_2/balance air. An overlay of gelatin was placed on the colony prior to processing for electron microscopy (Appendix II, Sect. 1). The electron micrograph shown is a longitudinal section through a *N. gonorrhoeae* colony containing *M. hominis*. Generally, the mycoplasmas are of smaller dimensions than the neisseria. At the higher magnification of the insert, the smooth external surface of the limiting membrane of the mycoplasma can be distinguished from the less regular outline of the cell wall of the neisseria. Arrows indicate some of the mycoplasmas. The magnification of A is x4,250 and the magnification of B is x19,915. The bars represent 1 μm.

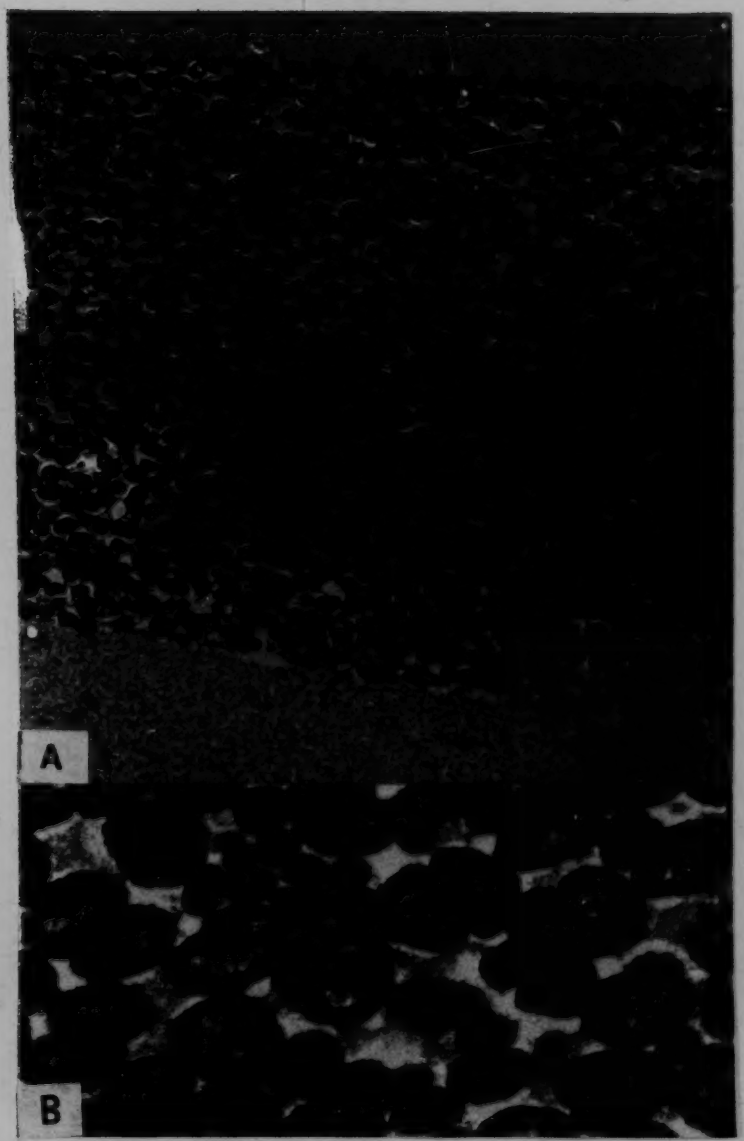
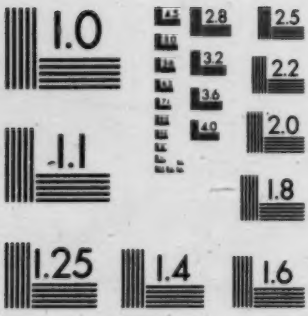

A
B

Figure 5 Transmission electron micrographs of the interrelated growth of a 48 hour old culture of Mycoplasma hominis strain M_5 and Neisseria gonorrhoeae strain C_5M.

The culture on GCS agar was incubated at 36°C for 48 hrs in 7% CO_2/balance air. The culture was prepared as described in Fig. 4. The mycoplasmas (indicated by the arrows) are more easily identified because there are larger numbers of them than in Fig. 4 and, unlike the neisseria, they still have an electron dense cytoplasm. In A the mycoplasmas can be found not only within the neisseria colony but also beneath the surface of the agar. B, taken from the colony-agar interface, contains degenerate neisseria cells, their electron density apparently lost through autolysis. The magnification in A is x4,250 and the magnification in B is x19,915. The bars represents 1 μm.

A
B



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

ct of Mycoplasma hominis on the auxotypes of
seria gonorrhoeae standard strains.

Auxotypes and strain designations[+]

	Subsequent passage number on :			
	GC agar		GCS agar	
Auxotype)	3	6	9	18
	N.R.*	P^-	P^-	P^-
	P^-	P^-	P^-	P^-

ts were established for proline (P), citrulline
ne (O), uracil (U), hypoxanthine (H) or the
non-requiring (N.R.).

eria were passaged first on GC agar for six
then on GCS agar for twelve passages. Each
after 24 hrs incubation at 36°C in 7 %
air. M. hominis was detected by microscopy,
ific colony fluorescence and/or growth in B
M. hominis was no longer detected in cultures
C_5M these cultures were designated C_3M^- and
ively.

ve than either LM or the colony fluorescence test for
ng M. hominis. All three methods were utilized to
the cultures throughout this experiment. The
es of the neisseria strains were checked after every
ransfers. The auxotype for both strains C_3M and C_5M
from non-requiring (N.R.) to proline-requiring (P^-)
ey became mycoplasma-free (Table 9). This change was
d after the third passage for strain C_5M and after
th passage for strain C_3M. The P^- auxotype of both
remained the same for at least twelve subsequent
s on GCS agar. The change in auxotype from N.R. to
r passaging on the serum-free GC agar has been
d twice with strains C_3M and C_5M; similar results
tained for both strains on all three occasions. The
f M. hominis from the N. gonorrhoeae cultures
ed with the neisseria changing from prototrophic to
phic (Table 9). If M. hominis was "cross-feeding"
sseria, it should have been possible to change the P^-
to protrophic strains by mixing them with M.
. The neisseria strains C_3M^- and C_5M^- from which
smas had been eliminated (and subsequently had become
types) were mixed with the corresponding M_3 and M_5
of M. hominis. Although mycoplasmal growth within

Plasmid profiles

The P^- auxotypes observed for both strains C_3M^- and were unchanged over multiple passages (Table 9). The lity of the altered auxotypes indicated possible ypic alterations. To determine if M. hominis had an on the plasmid profile of the neisseria, the plasmid les of the six stock cultures that had been passaged times on GCS agar were compared to the corresponding ns that had been passaged six times on GC agar and three times on GCS agar (Fig. 6). The C_5 auxotype ard strain had a 2.6 Mdal plasmid when cultivated alone 6, lane 2) or with the inadvertent (Fig. 6, lane 3) or tional (Fig. 6, lane 6) presence of M. hominis. ging the cultures on serum-free medium did not affect lasmid (Fig. 6, lanes 8, 9, & 12 respectively). The C_3 ype standard strain showed no plasmid when cultured S or GC agar (Fig. 6, lanes 4 & 10). Strain C_3M which ertently contained M. hominis, had a 2.6 Mdal plasmid 6, lane 5) that persisted after the strain had been ged on GC agar and was mycoplasma-negative (Fig. 6, 11). Neither the N. gonorrhoeae standard strain C_3 was intentionally co-cultivated with M_3 on either GCS agar (Fig. 6, lanes 7 and 13, respectively) nor pure res of M. hominis strain M_3 that had been isolated C_3M contained plasmids.

DS-PAGE protein profiles

Whole cell extracts of N. gonorrhoeae auxotype standard

e 6 Effect of *Mycoplasma hominis* on the plasmid content of *Neisseria gonorrhoeae*.

Lane 1 contains *E. coli* plasmid standards. The other contained DNA extracted from *N. gonorrhoeae*. Plasmid rom both *E.coli* and *N. gonorrhoeae* was extracted by the od discribed in Ch. II, Sect. 9.3.

	Passage Number:	
	Nine times on GCS agar	Six times on GC agar then three times GCS agar
re	Lane No.	Lane No.
	2	8
	3	9
	4	10
	5	11
M. hominis	6	12
M. hominis	7	13

se the samples used for electrophoresis were freshly red, the plasmid DNA was supercoiled and, therefore, ced a single band. No mycoplasmas were detected in any res after multiple passages on GC agar.

ains, with and without contaminating M. hominis, were
ected to electrophoresis on sodium dodecyl sulfate
acrylamide gels (SDS-PAGE) to determine if M. hominis
cted the protein-banding patterns of N. gonorrhoeae
. 7). The N. gonorrhoeae strains C_3 and C_5 (Fig. 7
es 2 and 6) had protein banding patterns which differed
those observed when M. hominis was present (Fig. 7
es 3 and 7). The most significant change observed was
alteration in apparent molecular weight (Mr) for the
coccal major outer membrane protein (P.I) of C_3 and C_5.
d on this gel, the Mr for the P.I of strain C_3 was
00 (Fig. 7 lane 2) and for strain C_5 was 36,500 (Fig. 7
e 6). When M. hominis co-existed with the neisseria
and C_5M), the Mr of P.I from both neisseria strains
36,800 (Fig. 7,lanes 3 and 7, respectively). When the
ominis was eliminated by passaging the contaminated
sseria on GC agar, the P.I protein of both N.
rrhoeae strains C_3M^- and C_5M^- had Mr of 37,500
ons (Fig. 7 lanes 4 and 8 respectively). The altered Mr
.I from both strains C_3M^- and C_5M^- did not change when
e strains were passaged on GCS agar. To determine if the
ered Mr of the P.I was due to M. hominis proteins, the
ein profile of the neisseria strains was subjected to
tern" immunoblotting (Ch. II, Sect. 4.2). The protein
ls obtained on SDS-PAGE were electrophoretically
sferred to nitrocellulose. All protein bands visualized

igure 7. Protein profile of Neisseria gonorrhoeae auxotype standard strains C_3 and C_5 with and without Mycoplasma hominis.

Auxotype standard strains with no mycoplasmas (C_3 and $_5$), standards with mycoplasmas (C_3M and C_5M) and standard trains that had been passaged on serum-free medium (GC gar) to eliminate the mycoplasmas (C_3M^- and C_5M^-) were ncubated on GCS agar at 36°C for about 24 hours. Whole ell extracts of these N. gonorrhoeae cultures were ubjected to electrophoresis on 12.5% sodium dodecyl ulfate polyacrylamide gels and then were stained with oomassie blue (Ch. II, Sect. 4.1)

ane	Sample
	Standard molecular weight markers.
C_3	N. gonorrhoeae (PCU).
C_3M	N. gonorrhoeae (N.R.) contaminated with M. hominis
C_3M^-	N. gonorrhoeae (P^-) that had been passaged on GC agar to eliminate M. hominis
M_3	M. hominis isolated from C_3M
C_5	N. gonorrhoeae (CUH^-)
C_5M	N. gonorrhoeae (N.R.) contaminated with M. hominis
C_5M^-	N. gonorrhoeae (P^-) that had been passaged on GC agar to eliminate M. hominis
M_5	M. hominis isolated from C_5M.

igure 8. Immunoblots of the protein profile of Neisseria gonorrhoeae auxotype standard strains C_3 and C_5 with and without Mycoplasma hominis.

Whole cell extracts of N. gonorrhoeae (described in ig. 7) were subjected to electrophoresis on 12.5% odium dodecyl sulfate polyacrylamide gels and then ransferred electrophoretically to nitrocellulose sheets. A) was stained for protein with amido black and (B) was eacted with rabbit anti-M. hominis strain 14027 serum and hen reacted with HRP-conjugated (goat) anti-rabbit mmunoglobulin (Ch. II, Sect. 4.2). The nitrocellulose heets were prepared from duplicate polyacrylamide gels ontaining the following samples:

ane	Sample
	Standard molecular weight markers.
B broth	This lane contained 1 µl of unconcentrated B broth in sample buffer
M_5	M. hominis isolated from C_5M
C_5M^-	N. gonorrhoeae; M. hominis had been eliminated by passage on GC agar
C_5M	N. gonorrhoeae contaminated with M. hominis
C_5	N. gonorrhoeae
M_3	M. hominis isolated from C_3M
C_3M^-	N. gonorrhoeae; M. hominis had been eliminated by passage on GC agar
C_3M	N. gonorrhoeae contaminated with M. hominis
0 C_3	N. gonorrhoeae
1	Standard molecular weight markers

1 2 3 4 5 6 7 8 9 10 11
MW
92,500
66,200
45,000
31,000
21,500
14,400
A

1 2 3 4 5 6 7 8 9 10 11
92,500
66,200
45,000
31,000
21,500
14,400
B

ate protein transfer. A duplicate blot was probed with
t anti-M. hominis strain 14027 serum (Fig 8B). Only
nole cell extracts of C_3M and C_5M contained components
reacted with M. hominis antiserum (Fig. 8B, lanes 3
, respectively).

scussion

Interrelated growth of genital mycoplasmas and N. rhoeae

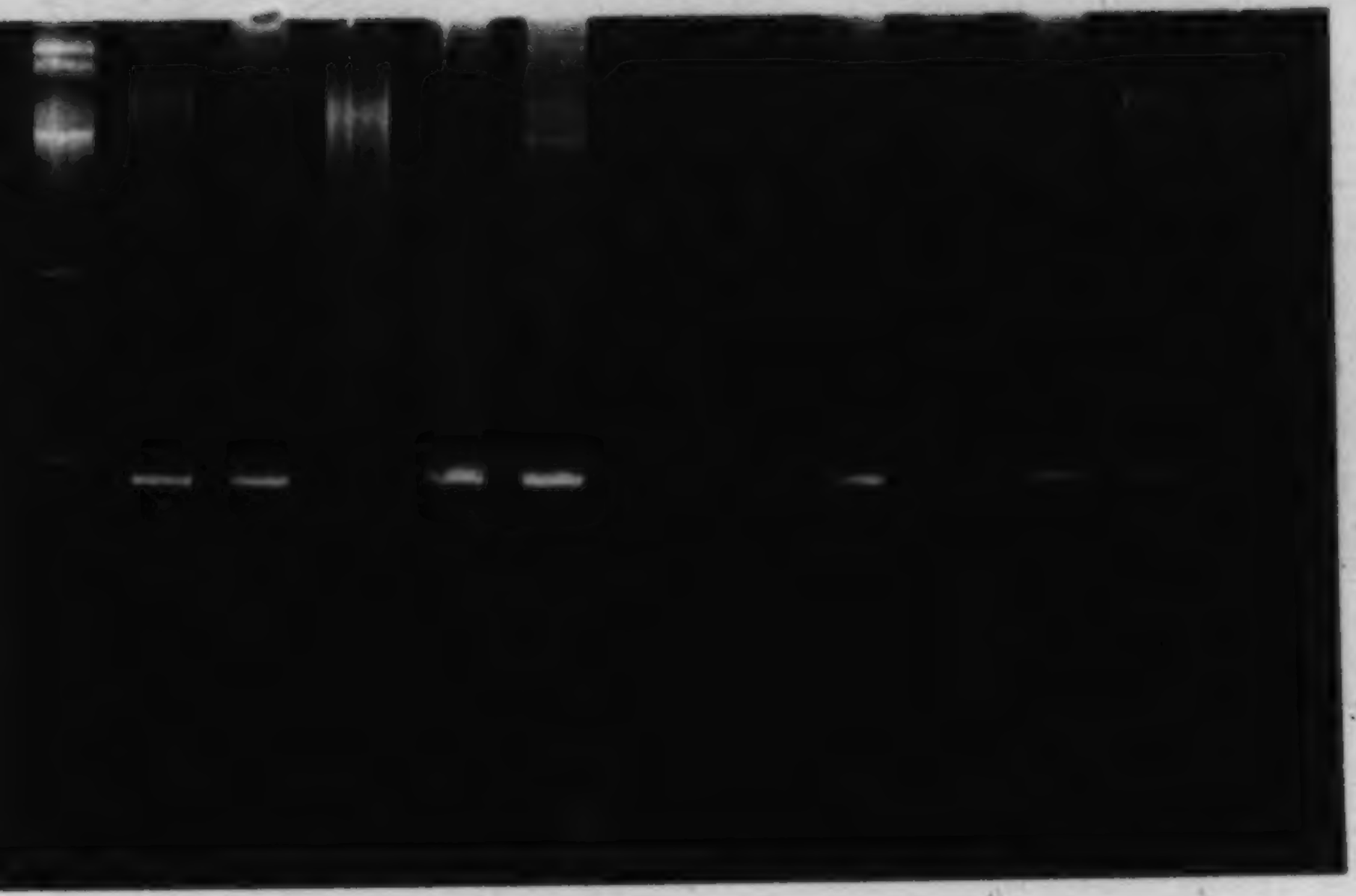
The results presented in this chapter (Tables 5 & 8,
1, & 2) support Brown and Haye's (1942) observation
apparently pure cultures of N. gonorrhoeae may
in mycoplasmas. Although mycoplasma contamination of
e cultures is well-documented and widely recognized,
o-existence of mycoplasmas with bacterial cultures has
given little attention. Mycoplasmas are frequently
nt on the mucosal surfaces of the human respiratory and
nital tracts. These serum-requiring organisms can grow
ny of the richly supplemented media designed for
dious bacteria. The present study examined
tes from urethral exudate from men and studied the
cts that interrelated growth might have on the
seria. In previous studies of males, genital
lasmas have been found to co-exist with N. gonorrhoeae
proximately one-third of the patients (Jahn et al 1982,
am et al 1972). Those studies utilized separate media
solation of mycoplasma and neisseria strains and thus

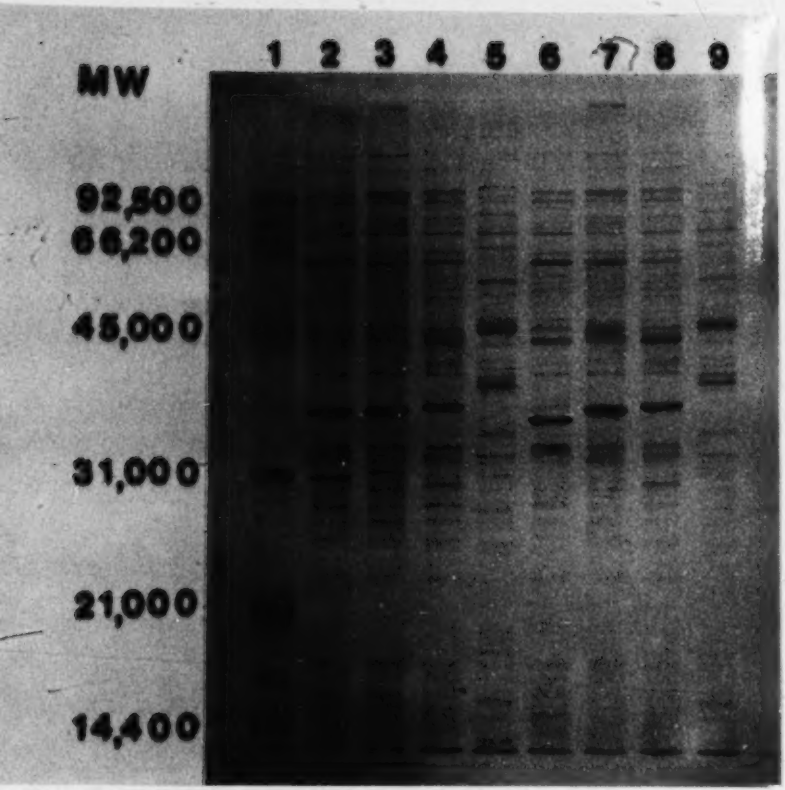
determined. The results presented in this chapter sho
that if serum components were present in the medium used
isolate N. gonorrhoeae from urethral exudate, then t
genital mycoplasmas (Ureaplasma urealyticum and Mycopla
hominis) were co-isolated in about 30% of the specime
(Table 5). This co-isolation incidence was lower than t
80% previously documented for women (Faur et al 1974) a
probably reflects the higher incidence of mycoplas
colonization of women (McCormack et al 1972, Markham et
1972, Taylor-Robinson & McCormack 1980a). In our study
urealyticum was isolated four-times as often as M. homi
from men with gonorrhea (Table 5). The ratio of
urealyticum to M. hominis observed in our study was simi
to that reported for isolates obtained from women (Faur
al 1974) and men (Csonka et al 1966, Jahn et al 1982) wi
gonorrhea .

Because of the prevalence of ureaplasmas co-isolat
with neisseria, these co-isolates were examined to determ
if there was any correlation between the ureaplasma seroty
and the neisseria auxotype. All of the wild-ty
ureaplasmas had multiple serological specificities (Tab
6) but this is expected for clinical isolates (Stemke
Robertson 1985). The overall prevalence of each ureapla
serovar in this study (Table 6) was similar to th
reported by Stemke and Robertson (1985) for both NGU a
healthy, control patients. Unlike Shepard and Luncefo

ains isolated from patients with gonorrhea b
er of isolates in their studies and in th
lyses difficult. Piot (1976) used the eigh
ng scheme and 4 of the 12 ureaplasma isolat
gonorrhea could not be typed. In this stud
U. urealyticum isolates tested could be typ
onstrating the value of the fourteen-memb
eme (Robertson & Stemke 1982) rather than t
erotyping scheme. No correlation was appare
rotype of U. urealyticum and the auxotype
d N. gonorrhoeae.

, and 3 support Faur's observation that t
rm "comet-shaped" colonies when growing with
e colonies. Fluorescence staining of t
growth established that both the "head" a
comet formations were composed of mycoplasm
ansmission electron micrographs of cros
olonies of N. gonorrhoeae infected with
4, & 5) showed mycoplasma cells that we
among the neisseria cells, throughout t
of the neisseria colony. It is apparent f
that subculture of this N. gonorrhoeae col
in the transfer of many mycoplasma cel
imately one-half of the N. gonorrhoeae stra


1 2 3 4 5 6 7 8 9 10 11 12 13


MW
1 2 3 4 5 6 7 8 9
92,500
66,200
45,000
31,000
21,000
14,400

sociation between ureaplasmas and neisseria is shown in ble 7. Even after six washes, ten times more ^{125}I-eaplasmas were detected in the N. gonorrhoeae pellet mpared to a ureaplasma control which contained no isseria (Table 7).

2 Effect of the co-existence of M. hominis on the auxotype N. gonorrhoeae

This aspect of the research involved N. gonorrhoeae andard strains rather than isolates from the study of ethral exudate. Genital mycoplasmas were not found in e original stock strains that were grown from lyophilized ltures and M. hominis was present in only some of the ltiple tubes of frozen stock cultures. Although the dium components were screened and additional lyophilized eparations of the auxotype standard strains were tested, ere is no satisfactory explanation regarding the source of hominis in the thawed stock cultures. Mixing pure N. norrhoeae with M. hominis did not result in an alteration the auxotype or protein profile. The strains were cloned fore I received them. Despite these shortcomings, it mains noteworthy that M. hominis could be associated with anges in the characteristics of certain strains of N. norrhoeae. A Steer's replicator was used for transferring

ifferent Neisseria strains became mixed. However, this unlikely because colonies on the auxotyping media did ppear to overlap. Also, this experiment was performed ree separate occasions and the same results were ed each time. Repeated testing demonstrated that the ent molecular weight (Mr) of the P.I of C_5M and C_3M ed upward and the auxotype changed from NR to P^- when ontaminating mycoplasmas were eliminated (Fig. 7 & 9, respectively). These results are the first ation that mycoplasmas may be associated with ations in the Mr of the P.I and the auxotype of N. rhoeae.

Examination of the immunoblots of contaminated eria strains showed components that reacted with M. is antiserum (Fig. 8). Some bands observed for M. is alone were not were not present in the mixed res. This may be due to differences in the number of minis in these preparations. When M. hominis was nated from C_3M and C_5M no immuno-reactive bands were ved. This supports the conclusion that M. hominis had eliminated. It is possible that a genitic alteration ccurred since neither the Mr of P.I nor the auxotype of or C_5M^- underwent any further change. Mechanisms by the wall-less mycoplasmas could contribute to ypic changes in a markedly different prokaryote, in case a bacterium with a cell wall, can not be readily

se permanent chromosomal aberrations in eukaryotic cells. so, piliated N. gonorrhoeae strains, unlike other teria, are constituitively competent for transformation, produce few or no restriction enzymes (Korch et al 3).

M. hominis, the species of concern in this study, may vide, in addition to its chromosome, DNA in plasmids uzias et al 1973, Harasawa & Barile 1983) or in virus-e particles (Robertson et al 1972). To determine if smids might be responsible for some of the the altered sseria characteristics, we investigated the plasmid tent of both the N. gonorrhoeae standard strains and the hominis contaminants. The PCU$^-$ strain used in this study tained no plasmids (Fig. 6). Dillon et al (1981) have onstrated that N. gonorrhoeae strains with PCU$^-$ otype characteristically lack plasmids. However, there evidence that plasmid-free PCU$^-$ strains contain omosomal sequences that are homologous to the 2.6 Mdal ptic plasmid frequently found in other N. gonorrhoeae ains (Bryne et al 1985). When the PCU$^-$ auxotype standard tained contaminating M. hominis, the auxotype was N.R. a 2.6 Mdal plasmid was detected (Table 8, Fig. 6). s plasmid persisted after the contaminating mycoplasmas been eliminated and the auxotype had changed to P$^-$. M. hominis strain isolated from C_3M had no detectable

s study is not known. Further genetic studies are uired to elucidate the mechanism(s) responsible for the erved changes. For instance, using the 2.6 Mdal plasmid a probe, it would be possible to determine if the PCU⁻ otroph in this study contained homologous chromosomal uences.

The Mr of P.I has normally been considered as a stable racteristic. Spontaneous mutants that showed changes in of P.I have been mentioned (Cannon & Sparling 1984) but culture conditions were not described. The only other lished report of a permanent change in the Mr of P.I was to genetic transformation (Hildebrandt et al 1978). ey showed that DNA from serum-resistant N. gonorrhoeae rains could transform serum-sensitive strains to serum-sistance. The Mr of the P.I protein of the transformed rains decreased by approximately 2500 (Hildebrandt et al 8). I did not attempt to determine if transformation of gonorrhoeae by M. hominis DNA was possible. Blake and schlich (1983) demonstrated that proteases could cleave e P.I of N. gonorrhoeae producing fragments of lower Mr. hominis produces proteases but whether these could affect e P.I of N. gonorrhoeae is unknown.

Repeatedly I was able to demonstrate that when the ntaminating mycoplasmas were eliminated by propagating the M and C_5M neisseria strains on serum-free GC agar, both rains became P⁻. Despite several passages of both rains on GCS agar, the auxotypes remained P⁻. The N.R. and

oratory Centre for Disease Control (Ottawa, Canada). A
ible explanation for these changes from NR to P^- is that
proline requirements of the neisseria were provided
ough cross-feeding by the M. hominis. M. hominis is
nolytic, producing ornithine in the log phase and
ulline in the lag phase of growth (Schimke & Barile
). Both glutamate and ornithine have been established
recursors of proline for other bacterial strains (Adams
ank 1980). M. hominis may provide proline or some
ursor such as ornithine to the neisseria strains which
ows them to survive on proline-deficient medium.
ough no auxotype changes were detected when M. hominis
mixed with N. gonorrhoeae it is possible that M. hominis
not in log phase growth and therefore would not produce
thine. The only other documented change in auxotype was
rted by Kenyon (1978). He found that N. gonorrhoeae
ins passaged in mice changed from P^- to PCU^-. Catlin &
(1978) also noted that arginine transformants (i.e.
ir of citrulline and ornithine requirements using
sformation with DNA from other N. gonorrhoeae strains)
only also lost their uracil requirement although there
been no selection for the latter trait. These studies
cate a close relationship between the arginine and
midine pathways similar to that known to exist for
ric bacteria (Catlin & Nash, 1978). Whether these

ginine, uracil and hypoxanthine requirements observed in is study is not known.

Both auxotype (Carifo and Catlin 1973, Hendry & Stewart 79, Copley et al 1983) and serotype (using P.I antigens) ang et al 1977, Copley et al 1983, Knapp et al 1984) have en proposed as suitable, stable phenotypes useful for idemiological studies. Both methods have been used to tablish the world-wide geographic distribution of various gonorrhoeae strains and to monitor their spread (Dillon d Pauze 1981, Ruckdeschel et al 1983, Copley and glestone 1983, Ansink-Schipper et al 1984, Knapp et al 84, Sandstrom et al 1984). The results presented in this esis raise questions regarding current methodologies. ny epidemiological studies have used wild-type strains of gonorrhoeae that were isolated, propagated and even metimes stored in medium that contained serum or its mponents (Catlin 1974, Hendry & Stewart 1979, Copley & glestone 1983, Nobel & Parekh 1983, Ruckdeschel et al 83, Ansink-Schipper et al 1984). While it is assumed at pure cultures of N. gonorrhoeae were used, no dication was given in these reports of any attempt to sure that the N. gonorrhoeae strains were mycoplasma-free. t this study demonstrates that about one-third of the N. norrhoeae isolates obtained from urethral exudate ntained genital mycoplasmas (Table 5). If undetected nital mycoplasmas were present in a culture, the termination of N. gonorrhoeae auxotype and, perhaps,

N.R. when contaminated with M. hominis (Table 8). Th
type change from N.R. to P⁻ occured after th
aminating M. hominis was eliminated from the N
rrhoeae auxotype standard strains (Table 9). In th
erlands (Ansink-Schipper et al 1984), German
deschel et al 1983) and the U.K. (Copley & Eggleston
), N.R. was the auxotype most commonly reported
729 (69%), 97/165 (59%) and 35/120 (29%), respectively
he N. gonorrhoeae strains in these studies had bee
aged on a serum-free medium before they were auxotyped
d the incidence of N.R. have been less?

The data presented in this chapter confirm that N
rrhoeae and genital mycoplasmas are frequently co
ated and can co-exist in vitro if serum is present i
medium. Workers need to be alerted to the frequen
solation of these two species and the need to assur
N. gonorrhoeae isolates are mycoplasma-free. While i
take time to investigate the provocative possibilitie
genital mycoplasmas may alter N. gonorrhoea
acteristics, the findings to date clearly present
ng argument for using serum-free medium for th
aration of pure cultures of neisseria strains.

RYOTIC CELLS. CHARACTERIZATION OF THE HELA 229 CELL

Introduction

Despite the abundance of information regarding the ation of U. urealyticum from the genital tract, the chment of ureaplasmas to eukaryotic cells has not been nsively studied. The few published reports involving ryotic cell lines were primarily attempts to establish ic infection of these cell cultures (Mazzali & Taylor-nson 1970, Masover et al 1976). One of the purposes of research was to investigate the attachment of plasmas to eukaryotic cells. Few methods have been rted for detection of ureaplasmas that are bound to ryotic cells. Viability assays have been used almost usively in prior studies (Mazzali & Taylor-Robinson , Masover et al 1976, Stalheim et al 1976, Kraus et al , Taylor-Robinson et al 1978, McGarrity 1985). It is erable to utilize more than one method to detect oorganisms that have attached to eukaryotic cells kowiak & Marling-Cason 1984). Fluorescence microscopy iodination of the ureaplasmas were studied to determine hey could be used, in addition to the CFU viability y, to monitor the attachment of ureaplasmas to HeLa s. The first goal was to establish that the U. lyticum strains used in this study could attach to the

HeLa 229 cells and the next was to determine the duration of the association. The physical conditions of the *in vitro* model (i.e. reaction volume, temperature and medium) had to be defined prior to studying attachment characteristics.

2. Detection of *U. urealyticum* attached to HeLa cells

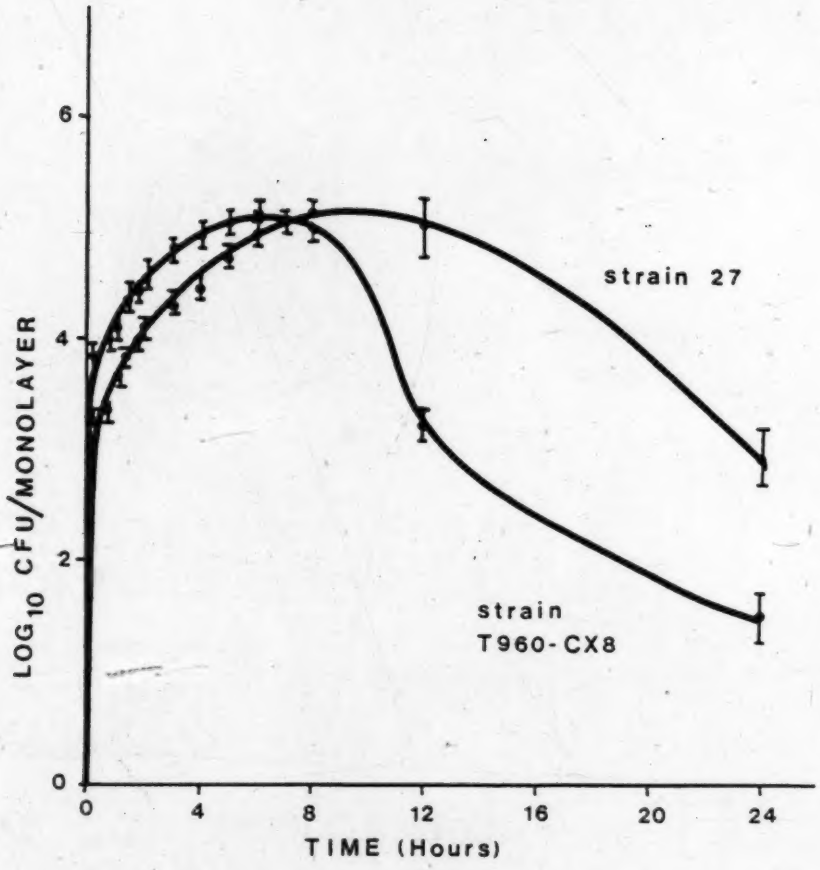
The HeLa cell line used in this study was grown at pH 6.8 by gradually decreasing the pH of the MEMS culture medium from pH 7.5 to pH 6.8 over four subcultures. Thereafter, the MEMS medium used in this study was at pH 6.8. *U. urealyticum* strain 27 (serotype 3) and strain T960-CX8 (serotype 8) were used for the attachment studies described in this chapter.

2.1 Colony forming unit assay

An experiment was performed to establish that the CFU assay could be used to detect ureaplasmas that had attached to HeLa cells and to determine the reproducibility of the CFU assay. The attachment of *U. urealyticum* to HeLa cells was monitored over 72 hrs by the CFU assay. Prior to enumeration, the monolayers were washed three times to ensure that all of the unattached ureaplasmas were removed. Because no viable ureaplasmas were detected at 48 hrs or 72 hrs post-infection, only the data for the first 24 hrs were shown (Fig. 9). The attachment of both ureaplasma strains over the first 30 mins was rapid, followed by a stage between 30 mins and 7 hrs that was less rapid. Maximum attachment of both strains was detected 8 hrs to 10 hrs post-infection; approximately 10^5 CFU of *U. urealyticum* were

Replicate monolayers were
MS-amp containing 2.6 x 1
rain 27 or 3.2 x 10^5 CFU
nolayers were incubated at 3
l then washed three times wi
eaplasmas attached per monol
U assay (Ch. II, Sect. 7.8.2).
s the mean $\pm$ the standard d
terminations.

inoculated with 1.0 ml of
10^5 CFU of U. urealyticum
of strain T960-CX8. The
50C for the times specified
th H buffer. The number of
ayer was measured using the
Each CFU value presented
eviation of four replicate


6
4
2
0
LOG10 CFU/MONOLAYER
strain 27
strain
T960-CX8
0
4
8
12
16
20
24
TIME (Hours)

eaplasma strains could attach to HeLa cells but t
ration of both the infections was less than 48 hrs. T
eoretical lower limit of detection for the CFU assay w
ureaplasmas bound per monolayer. This was calculated
e basis of a 0.025 ml aliquot producing one colony on
ar. Whenever possible, aliquots having between 30 to 3
lonies were counted.

2 Fluorescence microscopy

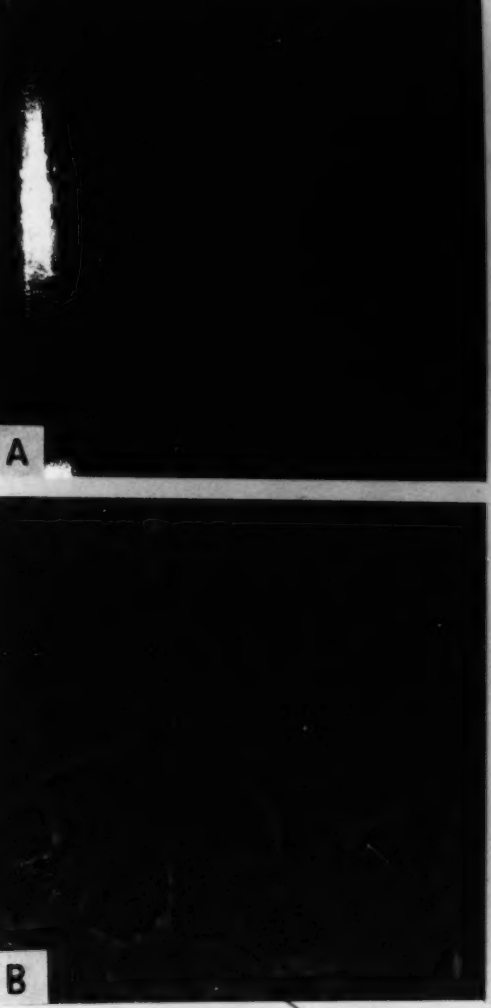
Visualization by microscopy is a valuable confirmati
at microorganisms have attached to the eukaryotic cell
rticularly when conditions preclude use of viabili
says. U. urealyticum generally are not visible by lig
croscopy. Fluorescence microscopy (FM) provided a mea
visualizing ureaplasmas that were attached to He
nolayers (Fig. 10). There was little backgrou
uorescence observed. The inoculum consisted of harvest
eaplasmas that had been sonicated (Ch. II, Sect. 7.
umps of fluorescent particles were only occasionally se
tached to the HeLa cells. At least 10^3 CCU
urealyticum attached per 10^5 HeLa cells were requir
r an unambiguous result by FM. The number of fluoresce
rticles attached to each HeLa cell was varied, with so
La cells having about twenty fluorescent particles a
hers having none (Fig. 10).

3 ^{125}I-radiolabel assay

The ureaplasmas were ^{125}Iodine-labelled by t

Figure 10 HeLa 229 monolayers which were infected with Ureaplasma urealyticum strain 27 and observed by light and fluorescence microscopy.

Replicate monolayers were inoculated with 1.0 ml of MEMS-amp containing 10^5 CCU of U. urealyticum strain 27. The monolayers were incubated at 36°C for 2 hrs, washed three times with H buffer and then fixed with acetone. The fixed monolayers were treated with rabbit anti-U. urealyticum strain 27 serum, followed by FITC (goat) anti-rabbit immunoglobulin (Ch. II Sect. 7.2.1). The uninfected control contained no fluorescent particles when observed by fluorescence microscopy (A). The same field as (A) is shown in (B) but using transmitted light. When infected monolayers were observed by fluorescence microscopy, fluorescent particles were observed (C). Arrows indicate fluorescent particles which would be near the 0.5 µm diameter of ureaplasmas. Panel (D), shows the same field as in (C) but viewed using transmitted light. The magnification is x480. The bar represents 10 µm.


A
B

C

D

had attached to HeLa cells since the specific activity of ^{125}I-ureaplasmas was 1 CPM per 1 to 10 ureaplasmas. CCU_{50} determinations performed in duplicate before and after iodination (1.4×10^9 CCU_{50}/ml and 1.1×10^9 CCU_{50}/ml respectively) indicated that the ureaplasmas remained viable despite ^{125}I on some of the tyrosine and tryptophan residues. Furthermore, the number of viable ureaplasmas was quite stable for up to 24 hrs when the iodinated ureaplasmas (^{125}I-ureaplasmas) were incubated at 4°C.

Since radioactivity was an indirect method for detecting ureaplasmas, it was necessary to verify that the ^{125}I did not dissociate from the ureaplasmas and that the radioactivity detected on infected HeLa cells represented bound ureaplasmas. About 10% of the ^{125}I in the iodinated ureaplasma suspension was not protein- associated (Ch. II, Sect. 7.5) and would be present in all suspensions. In the presence of serum the amount of ^{125}I that could not be sedimented was greater than the expected 10% that was detected in the H buffer control (Fig. 11). The dissociation of ^{125}I was dependent upon the type and concentration of serum added, with normal rabbit serum causing the greatest amount of dissociation (Fig. 11). All subsequent attachment assays were performed in H buffer.

FM confirmed that the ^{125}I-ureaplasmas attached to HeLa cells. If there are a finite number of binding sites

strain 27 in rabbit ser

4°C.

Normal rabbit and horse serum

then ^{125}I-ureaplasmas were in

al concentration of 8.1 x 10^5

ivity was 8.1 CPM per CCU of

quots of the suspensions were in

ropriate intervals the ^{125}I-ureap

centrifugation at 14,000 rpm for

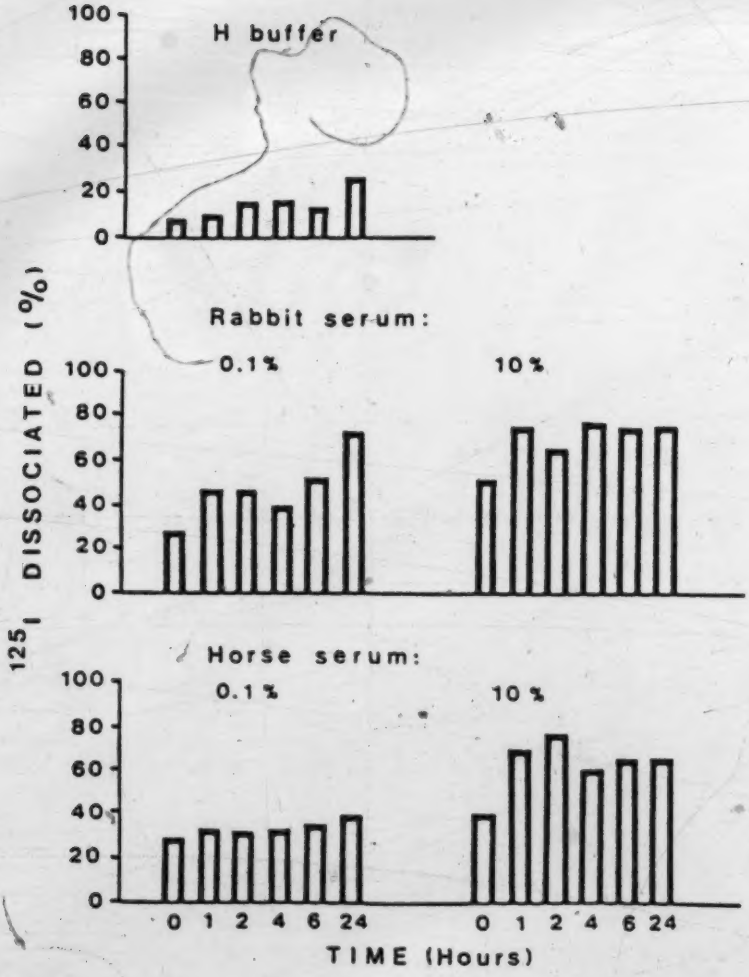
rofuge and then the amount of ^{12}

id was measured. Each CPM value

n of duplicate determinations.

rum and horse serum at

were added to H buffer
troduced to produce a
CPM/ml (the specific
ureaplasmas). One ml
cubated at 4°C. After
lasmas were sedimented
5 min in an Eppendorf
^{125}I in the supernatant
in the histogram is the


H buffer
Rabbit serum:
0.1%
10%
Horse serum:
0.1%
10%
100
80
60
40
20
0
^{125}I DISSOCIATED (%)
0 1 2 4 6 24
0 1 2 4 6 24
TIME (Hours)

n the HeLa cell surface then the addition of unlabelled reaplasmas to ^{125}I-labelled ureaplasmas should decrease ne amount of radioactivity that binds to the HeLa cell. If aturation kinetics have been achieved than there should be direct inverse relationship between the amount of nlabelled ureaplasmas added and the amount of radioactivity nat binds per monolayer. Unlabelled ureaplasmas could ompete with ^{125}I-labelled ureaplasmas for binding sites n the HeLa cells (Table 10). As more unlabelled reaplasmas were added to a constant number of ^{125}I-reaplasmas, less radioactivity bound to the monolayer Table 10). When the ratio of unlabelled to labelled reaplasmas was 200:1 the number of bound CPM was reduced y 37% and 40% at 36°C and 4°C, respectively (Table 10). ne amount of radioactivity that bound to the HeLa cells was ecreased but not completely inhibited by the addition of nlabelled ureaplasmas.

To determine how the two enumeration methods compared, oth the CFU and the IR assay were used to enumerate eplicate samples (Fig. 12). Since the specific activity of ne inoculum used for this experiment was 1 CPM per 10 CFU; or 10 CFU bound there should be 1 CPM detected. The actual atio of CFU to CPM bound was approximately 10 and therefore upported the specificity of the IR assay. The association ime for subsequent analyses was set as 1 hr because the ercentage of inoculated ureaplasmas which bound in this ime could be readily determined by both the CFU and IR

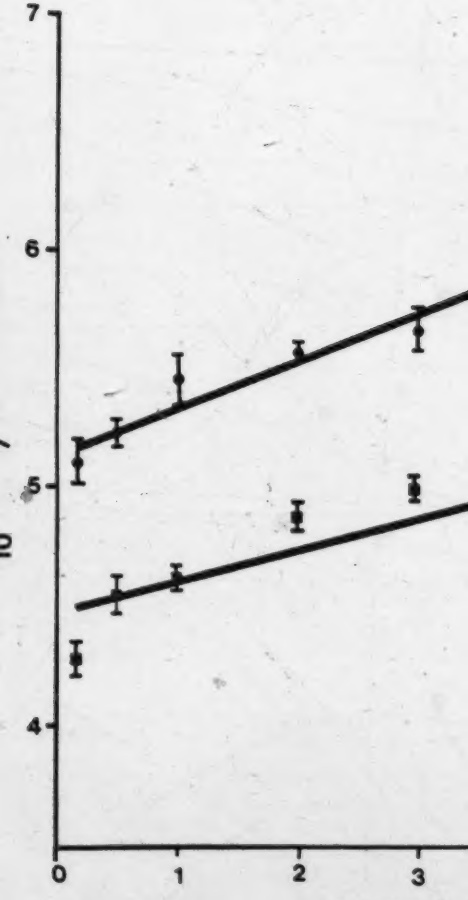
10 Inhibition of ^{125}I-*Ureaplasma urealyticum* strain 27 binding to HeLa 229 cells by unlabelled *U. urealyticum* strain 27 cells.

Ratio of unlabelled/ labelled cells	CPM bound/monolayer*			
	36°C	% reduction	4°C	% reduction
0:1 (control)	15,427		10,342	
10:1	11,559	(25%)	8,397	(19%)
20:1	11,488	(26%)	7,878	(24%)
100:1	10,407	(33%)	7,606	(27%)
200:1	9,666	(37%)	6,209	(40%)

* The test solutions were all prepared using H buffer containing 350,000 CPM of ^{125}I-ureaplasmas / ml (specific activity was 1.4 CPM per CCU of ureaplasmas) and the ratio of unlabelled ureaplasmas was as indicated. Replicate monolayers were inoculated with 0.2 ml of the test solutions (i.e. 70,000 CPM inoculated / monolayer). The monolayers were incubated at the indicated temperatures for 1 hr and then the amount of radioactivity that had attached per monolayer was determined (Ch. II, Sect. 7.8.3). Each CPM value was the mean of duplicate determinations.

measured by the CFU and IR assays

Replicate monolayers were inoculated with 0.2 ml of H buffer containing 8.7 x 10^5 CPM of ^{125}I-U.urealyticum strain 27 (specific activity of 1.1 CPM per 10 CFU of ureaplasmas). The monolayers were incubated at 4°C for the times specified and then washed three times with H buffer. The number of bound ureaplasmas was measured by the CFU and IR assays (Ch. II, Sect. 7.8.2 & 7.8.3, respectively). Each CFU value was the mean ± the standard deviation of four replicate determinations and each CPM value was the mean ± the standard deviation of duplicate determinations. The data were analyzed using linear regression. The r^2 value (coefficient of determination) for the CFU data was 0.95 and for the IR data was 0.87. The r^2 value is the measure of a linear relationship between two variables x and y.


7
6
5
4
0
1
2
3
TIME (H

CFU
CPM
7
6
5
4
LOG10 CPM/MONOLAYER
4
5
6
ours)

3. Duration of U. urealyticum strain 27 association with HeLa 229 monolayers.

These experiments were designed to determine if chronic ureaplasma infection of HeLa cells could be established. The monolayers were examined to determine the effect of passaging on the duration of ureaplasma association with the HeLa monolayers. All long term experiments were performed at 36°C. The medium used was MEMS-amp.

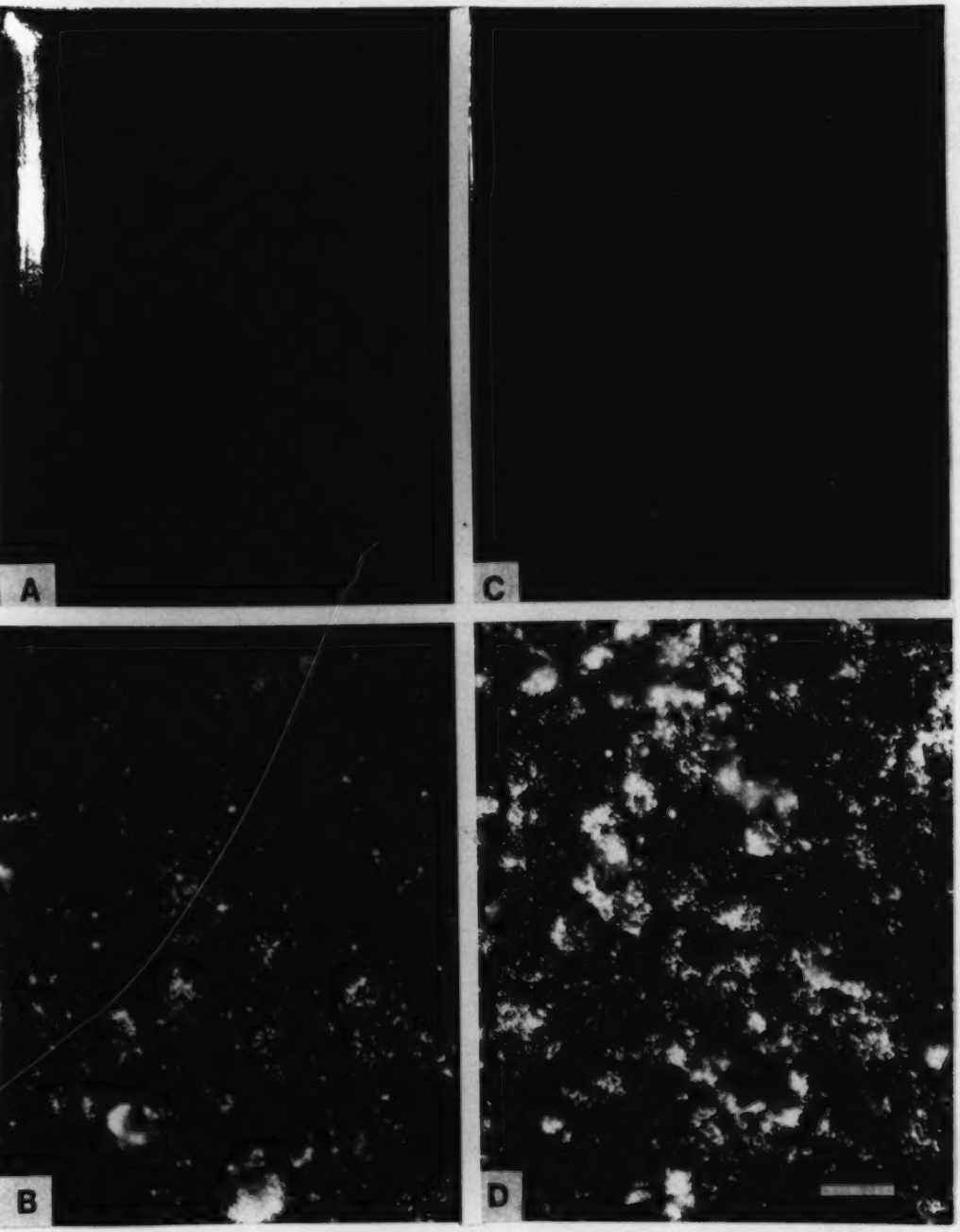
3.1 Non-passaged monolayers

The initial CFU attachment experiments indicated that neither of the ureaplasma strains tested remained viable for extended lengths of time when the infected HeLa monolayers were not passaged (Fig. 9). FM was also used to determine the length of ureaplasma association with non-passaged HeLa cells (Fig. 13). No localization of the fluorescent particles on the cells was observed over the 5 days of incubation (Fig. 13). Although the total number of fluorescent particles appeared to have increased by day 5 compared to those seen on day 1, no viable ureaplasmas were detected 2 days post-infection.

3.2 Passaged monolayers

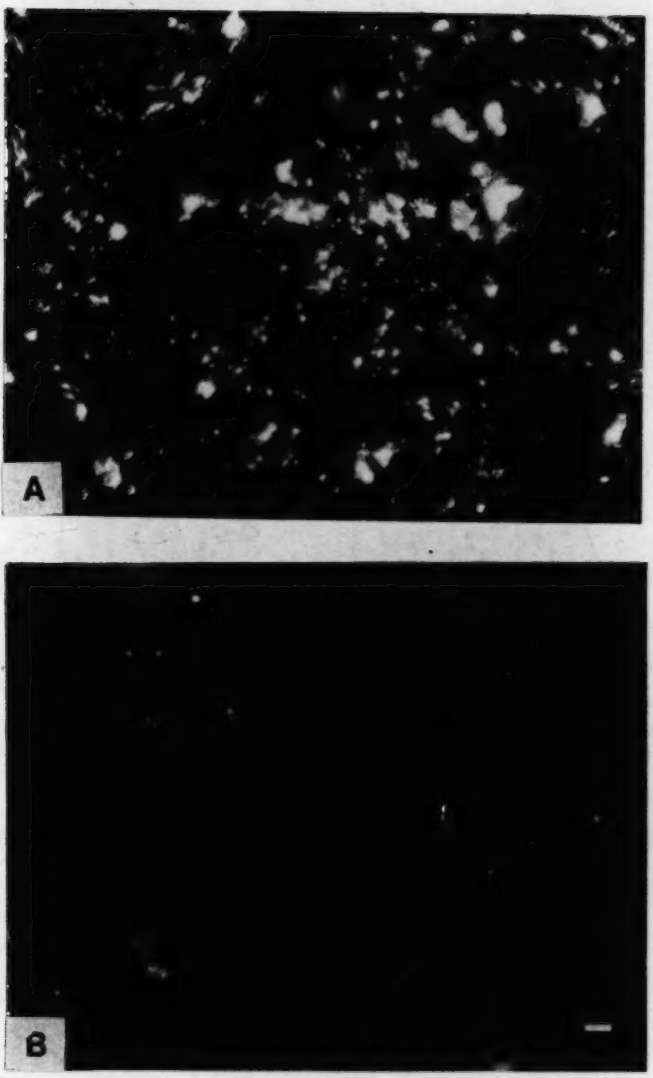
The following experiments were designed to determine if passaging the monolayers affected the distribution or duration of fluorescent particles on the HeLa monolayer. The effect of gentamicin was also examined to determine if a bactericidal antibiotic could affect the attachment or the

Replicate monolayers on 13 mm diameter coverslips were inoculated with 1.0 ml of MEMS-amp containing 10^8 CCU of U. urealyticum strain 27. The monolayers were incubated at 36°C. At appropriate intervals the monolayers on coverslips were washed three times with H buffer then stained for FM as described in Ch. II, Sect. 7.2. The ureaplasma-free controls for 6 hrs and 5 days post-infection are shown in (A) and (C), respectively. Many fluorescent particles cover the monolayer 6 hrs post-infection (B). The distribution of fluorescent particles observed 5 days post-infection (D) was similar to that seen 6 hrs post-infection (B). The magnification is x230. The bar represents 50 µm.


A
C
B
D

infected with U. urealyticum strain 27 on the distribution of fluorescent particles.

A 25 cm^3 flask of HeLa cells was inoculated with MEMS-amp containing 5 x 10^8 CCU of U. urealyticum strain 27. The flask was incubated at 36°C and was subcultured on day 2 and again on day 7 post-infection. Numerous fluorescent particles are apparent over the entire surface of a monolayer on a 13 mm coverslip that was withdrawn 1 day post-infection (A). On day 8 post-infection the fluorescent particles were occasionally localized in the cytoplasmic area of the HeLa cells (B). The magnification is x360. The bar represents 10 µm.


A
B

duration of ureaplasma association with HeLa cells. Gentamicin at a concentration of 50 µg/ml was used because this concentration would not affect the HeLa cells (Fischer 1975) whereas for U. urealyticum strain 27 the minimum inhibitory concentration was 8 µg/ml. When infected monolayers were passaged, the number of fluorescent particles gradually decreased until no fluorescent particles were detected. Occasionally cytoplasmic localization of the fluorescent particles occured (Fig. 14). Although no viable ureaplasmas were detected, the fluorescent particles observed 8 days post-infection (Fig. 14 B) appeared similar in size and staining intensity to those observed 1 day post-infection (Fig. 14 A).

When compared to MEM-amp, the addition of gentamicin decreased the length of time that viable ureaplasmas were detected but did not alter the duration or amount of fluorescence observed (Table 11). Fluorescent particles were consistently detected on the infected HeLa monolayers for 5 to 7 days after the viability assay for ureaplasmas became negative.

Replicates of the samples described in Table 11 were tested by the "Western" immunoblotting methodology to determine if this method could be adapted to detect ureaplasma antigens in infected HeLa cells. Although reactive bands were observed (Fig. 15), FM was still positive after the immunoblot became negative (Table 11, and Fig. 15, respectively).

urealyticum strain 27 with passaged HeLa 229 monolayers.

Sub-culture	Days post-infection	Titre* (CCU/ monolayer) MEMS-gent	Titre* (CCU/ monolayer) MEMS-amp	Fluorescence** MEMS-gent	Fluorescence** MEMS-amp
	1	10^5	10^7	++++	++++
--->	2	10^1	10^3	++++	++++
	3	0	10^1	+++	+++
--->	6	0	0	++	ND
	7	0	0	+	+
--->	14	0	0	±	±
	15	0	0	-	-

Non-infected control monolayers grown in MEMS-gent or MEMS-amp produced no colour change when subcultured into B broth and no fluorescence was observed.

* The inoculum was 10^8 CCU of ureaplasmas per monolayer. The monolayers were subcultured and tested as described in Ch. II, Sect. 7.9.

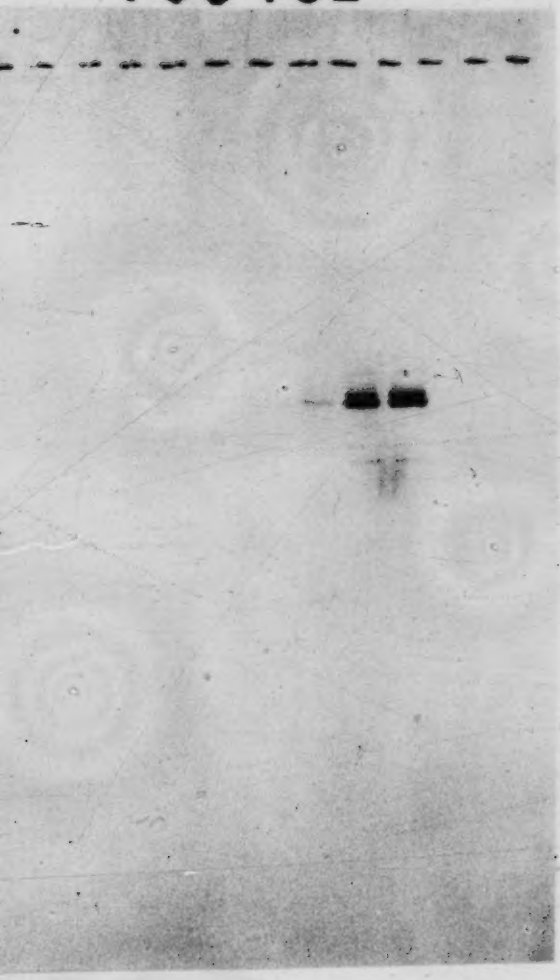
** "+" denotes positive fluorescence, "-" denotes a negative reaction, and "ND" denotes that the test was not done.

----> Arrows indicate the days post-infection that the HeLa cells were subcultured.

strain 27.

The samples used were replicates of those described in able 11 for MEMS-amp. The HeLa cells were sonically emoved from the coverslips, sedimented by centrifugation, nd the pellet processed for SDS-PAGE and Western mmunoblotting (Ch. II, Sect. 4.1 & 4.2, respectively). he samples were nonreduced and were electrophoresed on a 2.5% polyacrylamide gel. No reactive bands were observed or any of the mycoplasma-free monolayers.

ane number	Days post-infection
	1
	2
	3
	6
	7
	14
	15


7654321

4. Characterization of attachment assay conditions

The assay volume, temperature and medium were each examined to determine the standard attachment conditions which are described in Ch. II, Sect. 7.8. The CFU assay (Ch. II, Sect. 7.8.2) was used for enumerating the number of viable ureaplasmas that attached to HeLa cells. U. urealyticum strains 27 and T960-CX8 were suspended in 0.2 ml (the minimum volume required to completely cover the monolayer) or 1.0 ml of MEMS. The number of ureaplasmas which attached to the HeLa monolayers was monitored by the CFU assay (Fig. 16). To facilitate comparison, the data were transformed to Log_{10}, and analyzed using linear regression. Because the same total number of ureaplasmas was present in both of the reaction volumes tested, the ureaplasma concentration was five-times higher in the 0.2 ml than in the 1.0 ml volume. Both ureaplasma strains behaved similarly; attachment was approximately four-fold greater at 20 mins post-infection for the higher concentration of ureaplasmas (Fig. 16) although this diminished slightly over the period of incubation.

The effect of temperature on attachment of ureaplasmas was tested using the CFU assay (Fig. 17). Similar results were obtained for both U. urealyticum strains 27 and T960-CX8; attachment occurred at both 36°C and 4°C. At 120 mins post-infection about 10 times more ureaplasmas attached at 36°C than at 4°C (Fig. 17). The solutions examined for possible use in the attachment assays included: MEMS, B

re 16 The effect of ureaplasma concentration on the attachment of Ureaplasma urealyticum strains 27 and T960-CX8 to HeLa 229 cells.

Replicate monolayers were inoculated with either 1.5 x CFU of U. urealyticum strain T960-CX8 (A) or 1.5 x 10^5 of strain 27 (B). The inocula were suspended in 0.2 or ml of MEMS-amp. The monolayers were incubated at 36°C the times indicated. The CFU assay was used to erate the number of attached ureaplasmas (Ch. II, Sect. 2). Each CFU value is the mean ± the standard ation of four replicate determinations. The data were yzed using linear regression. For the strain T960-CX8 the r^2 values are 0.99 and 0.96 for the 0.2 ml and 1.0 reaction volumes, respectively. For the strain 27 data r^2 values are 0.88 and 0.96 for the 0.2 ml and 1.0 ml tion volumes, respectively.

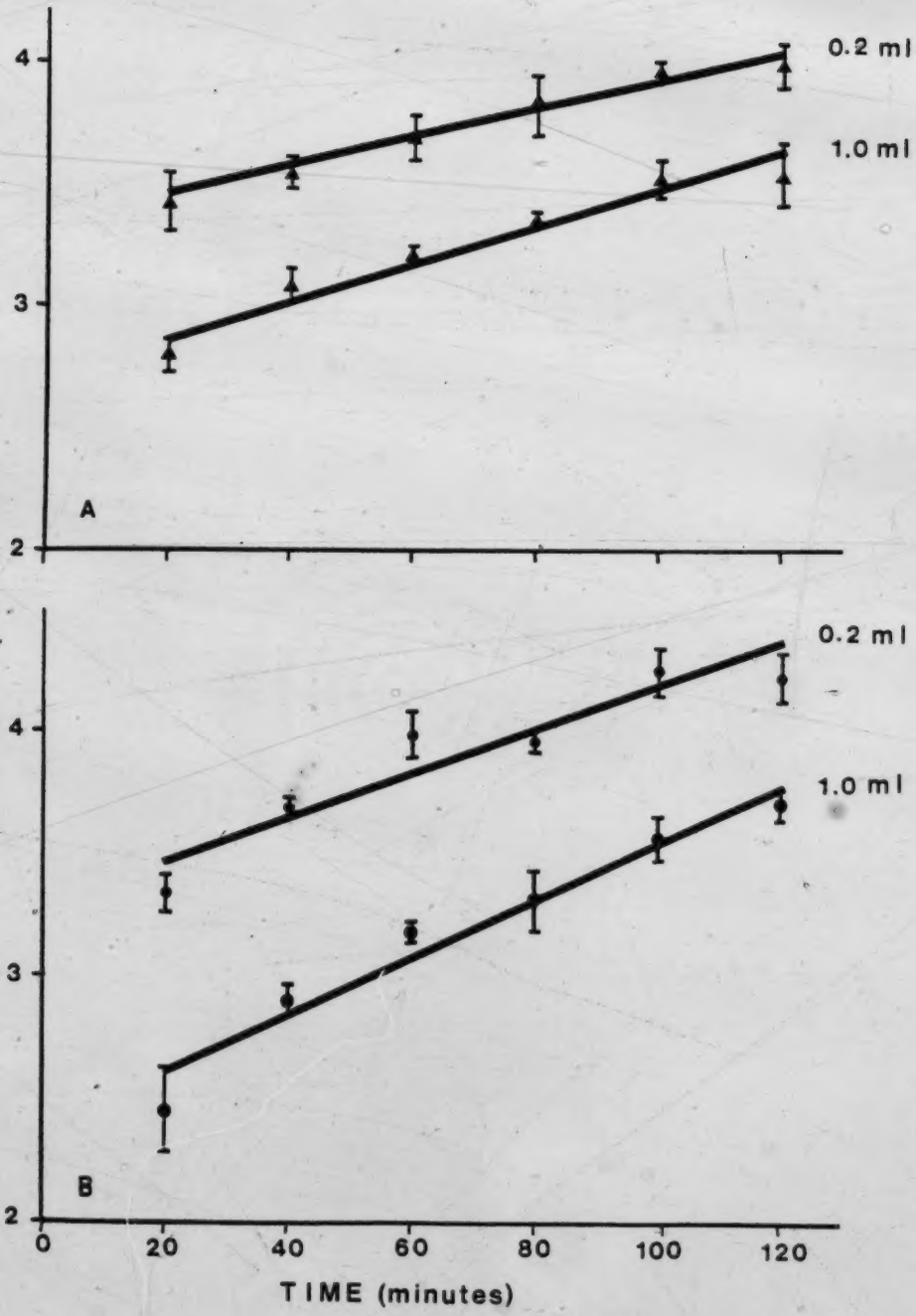
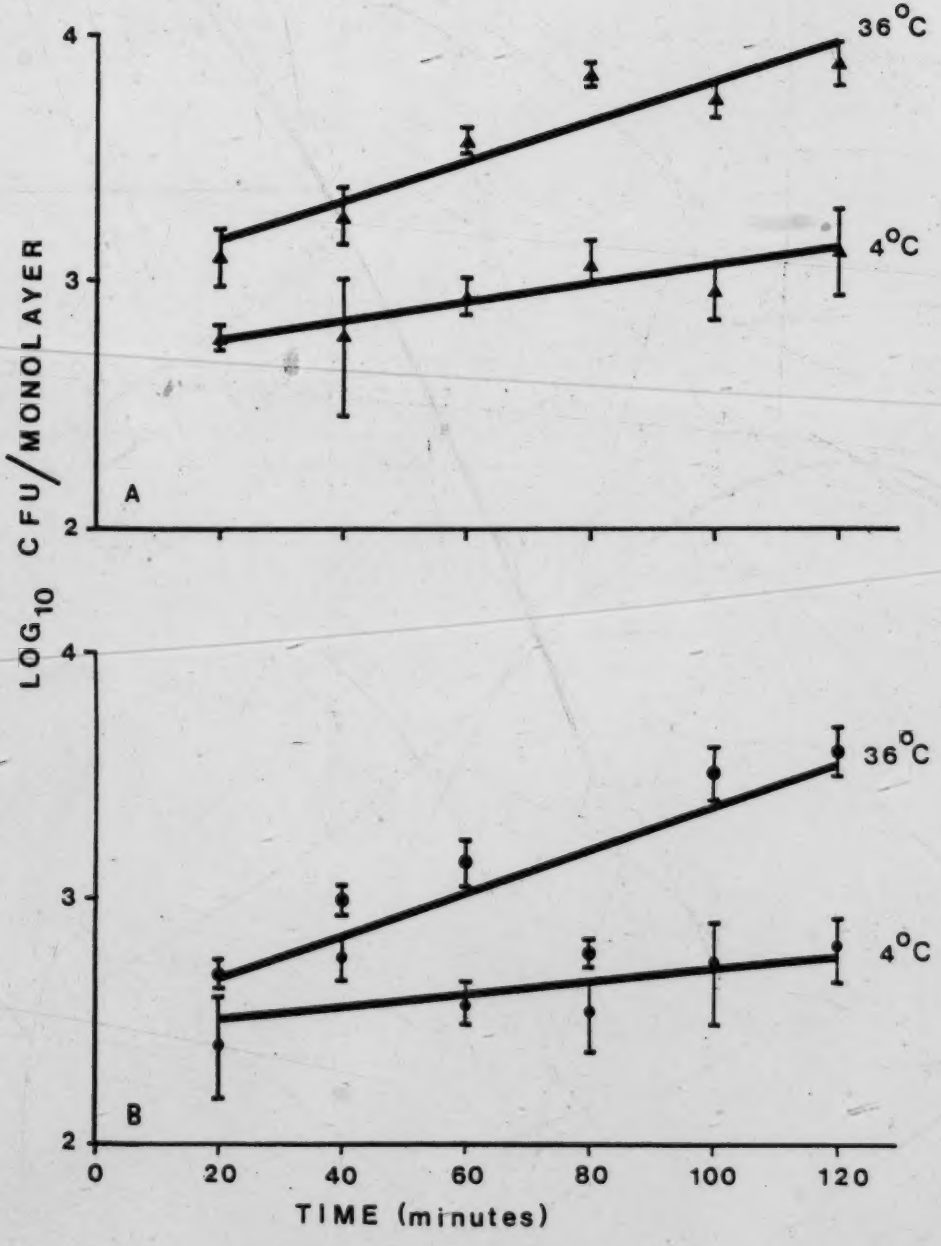

4
3
2
A
0.2 ml
1.0 ml
4
3
2
B
0.2 ml
1.0 ml
0
20
40
60
80
100
120
TIME (minutes)

Figure 1. The effect of incubation at 4°C and 36°C on the attachment of Ureaplasma urealyticum strains 27 and T960-CX8 to HeLa 229 cells.

Replicate monolayers were inoculated with 1.0 ml of MEMS-amp containing either 2.5×10^5 CFU of U. urealyticum strain T960-CX8 (A) or 6.8×10^4 CFU of strain 27 (B). The monolayers were incubated at 36°C or 4°C and then the CFU assay used to enumerate the number of attached ureaplasmas (Ch. II, Sect. 7.8.2). Each CFU value is the mean $\pm$ the standard deviation of four replicate determinations. The data were analyzed using linear regression. For the strain T960-CX8 data (A) the r^2 values are 0.87 and 0.85 at 36°C and 4°C, respectively. For strain 27 (B) the r^2 values are 0.66 and 0.44 at 36°C and 4°C, respectively.


4
3
2
36°C
4°C
A
LOG10 CFU/MONOLAYER
4
3
2
36°C
4°C
B
0
20
40
60
80
100
120
TIME (minutes)

2 Survival of Ureaplasma urealyticum strain 27 in H buffer at 36°C and 4°C.

me after oculation (hrs)	Titre of U. urealyticum* (CCU_{50} X 10^7/ml)	
	36°C	4°C+
0	3.9	3.9
1	1.2	4.5
2	2.1	2.8
4	1.8	1.7
6	1.6	1.7
24	**	1.4

Each value represents the mean of duplicate determinations.

When the data were analyzed by the two tailed Student t test, at the .050 level of significance, there was no significant difference in the titre obtained at the start of the experiment and those obtained at subsequent times over the 24 hrs tested.

Viability was checked by subculturing 0.2 ml of the lowest dilution into 5 ml of B broth. If there was no colour change after 4 days incubation, the titre was recorded as < 5 organisms per ml.

broth, and H buffer. For attachment assays of less than 6 hrs duration, the assay medium used was H buffer. A Hepes buffer (H buffer, Appendix I, Sect. 6) was chosen for the attachment assays because it is nontoxic for both tissue culture cells (Good et al 1966) and ureaplasmas (Manchee & Taylor-Robinson 1969b). The effect of H buffer on the viability of U. urealyticum was determined by measuring the CCU_{50} of ureaplasmas incubated in this buffer (Table 12). The number of viable cells decreased over 24 hrs, but at 4°C the decrease was not considered to be significant (Table 12). This non-nutritive buffer reduced the possibility that ureaplasma multiplication would interfere with interpretation of the results of the CFU attachment assays. B broth was inappropriate for experiments of long-term association because the ureaplasmas could multiply and the medium was unsuitable for HeLa cell growth. MEMS-amp at pH 6.8 was used for experiments of 24 hrs and longer.

5. Discussion

5.1 Duration of ureaplasma infection

An important consideration in these attachment studies was the choice of U. urealyticum strain. The use of wild-type isolates of unknown serotype (Shepard 1957, Mazzali & Taylor-Robinson 1971, Taylor-Robinson & Carney 1974) makes comparison of results difficult. The type strain T960 serotype 8 (Shepard et al 1974) has been used (Masover et al 1976a, McGarrity 1985) to study attachment of U. urealyticum

to eukaryotic cells. My initial characterizatio
experiments utilized U. urealyticum strains 27 (serotype 3
and T960-CX8 (serotype 8). The attachment of both U
urealyticum strains was readily monitored using the HeL
cell model (Figs. 9, 12, 16 & 17). Robertson and Stemk
(1985) reported that for symptomatic males, U. urealyticu
strains with the serotype 3 determinant were isolated mor
frequently (28/214) than were those of serotype 8 (9/214)
U. urealyticum strain 27 (the serotype 3 standard) has bee
shown to haemadsorb to guinea pig erythrocytes whereas th
strain T960 (the serotype 8 standard) does not (Blac
1973). Based upon its ability to haemadsorb and it
incidence in urethral exudate, U. urealyticum strain 27 wa
used for all subsequent attachment studies.

Although U. urealyticum strains 27 and T960-CX8 attac
to HeLa cells, the results in Fig. 9 and Table 11 suppor
previous reports that viable ureaplasmas can be detected fo
only short periods (e.g. 24 hrs to 48 hrs) post-infectio
(Mazzali & Taylor-Robinson 1971, Masover et al 1976a
McGarrity (1985), on the other hand, has produced infectio
of HeLa cell cultures. He reported that the ureaplasm
strain T960 survived in HeLa cells and could be maintaine
for at least five passages. The differences in survival ma
reflect differences in the cell line or culture medium. U
urealyticum strain T960 could survive up to 6 days at 36°
in the Dulbecco's minimum essential medium that McGarrit
(1985) utilized, whereas strains 27 and T960-CX8 used in m

research did not survive for even 24 hrs in the MEMS used for tissue culture. Indeed, the prolonged survival of ureaplasmas in Dulbecco's minimum essential medium at 36°C reported by McGarrity (1985) is in contrast to the rapid decline of ureaplasmas when incubated at 36°C in other broths designed for growth of mycoplasmas (Manchee & Taylor-Robinson 1969). DMEM is a richer medium than MEMS since it contains the additional amino acid serine as well as ferric nitrate and sodium pyruvate. Iron is essential for all bacteria (Finkelstein et al 1983); its role in ureaplasma metabolism is unknown. It is possible that the presence of ferric nitrate in DMEM facilitates the survival of ureaplasmas in vitro.

Although no viable ureaplasmas could be detected 48 hrs to 72 hrs post-infection, the FM assay remained positive for 5 days to 7 days post-infection (Table 11). The apparent distribution of the fluorescent particles 5 days post-infection was unchanged (Figs 13 & 14). It is possible that the fluorescent particles were non-viable ureaplasmas, alternatively they may be viable but non-cultivable on in vitro media. Masover et al (1976a) suggested that viable ureaplasmas may be present but undetectable by the currently available viability tests. McGarrity (1985) also reported that association with HeLa cells altered the ability of U. urealyticum strain T960 to grow on some types of synthetic medium. The results presented in this thesis indicate that ureaplasmal antigens remained associated with HeLa cells for

luorescent particles were viable ureaplasmas.

While the distribution of fluorescent particles was enerally uniform in non-passaged HeLa cell cultures (Fig. 3), passaging the monolayers resulted occasionally in ocalization of the fluorescent particles in the cytoplasmic rea of the eukaryotic cells (Fig. 14). To my knowledge no tudies have used immunofluorescence to study the long erm localization of ureaplasmas in infected cell culture. cGarrity (1985) used the immunoperoxidase method to monitor reaplasmas attached to HeLa cells. When he used an noculum of 10^3 CCU/ml, the ureaplasmas multiplied up to 10^5 CU/ml in the supernatant fluid and produced micro-colonies n only some of the HeLa cells. Micro-colony formation was ot evaluated in my experiments since ureaplasma ultiplication was not detected by the CCU or CFU assays.

The addition of gentamicin decreased the length of time hat viable ureaplasmas could be detected but did not revent ureaplasmas from attaching to HeLa cells (Table 11). hen ureaplasmas were incubated for about 18 hrs in MEMS ontaining 50 µg/ml gentamicin, viable ureaplasmas could not e detected. However, if HeLa cells were also present then iable ureaplasmas that had attached to the eukaryotic cells ould be detected for up to 48 hrs post-infection (Table 1). Since gentamicin does not enter eukaryotic cells Vandaux & Waldvogel 1979) the HeLa cell may protect the reaplasmas from the bactericidal effect of the antibiotic

Stanbridge (1971).

Components of infected HeLa cells that reacted with anti-U. urealyticum strain 27 serum could be detected using the "Western" immunoblotting technique (Fig. 15). Only about five immunoreactive bands were observed. This result differed from whole cell suspensions that displayed about thirty bands (Fig. 25) and may simply reflect lower numbers of ureaplasmas in the infected cell culture.

These studies demonstrated that ureaplasmas attached to HeLa cells but that the duration of U. urealyticum strains 27 and T960-CX8 were relatively short since viable ureaplasmas could not be detected 3 to 4 days post-infection. Therefore, studying the attachment characteristics of ureaplasmas was feasible using the HeLa cell model whereas studying chronic infection was not possible.

5.2 Characterization of the HeLa 229 attachment model

Having determined that it was feasible to study the attachment of ureaplasmas to HeLa cells, standard conditions were established. The importance of the pH of the milieu of the attachment models used to study ureaplasma attachment to eukaryotic cells has received little attention. Other in vitro models (Manchee and Taylor-Robinson 1969b, Mazzali et al 1971, Masover et al 1976a, Stalheim et al 1976, McGarrity 1985) monitored attachment at pH 7.0 to 7.5. The pH of the uninfected female vagina ranges from 4 to 4.5 (Holmes et al 1985) while the pH of the

nhabit such an environment, the HeLa cells that were us
n this study were grown at the lowest pH where good grow
ould be obtained, i.e. pH 6.8. The ability of HeLa cel
o grow in a slightly acidic environment (Eagle 196
trengthens the value of this model for studies
reaplasma attachment.

All of the previous studies on attachment of
realyticum to eukaryotic cells were performed only at 36
Manchee & Taylor-Robertson 1969, Mazzali & Taylor-Robins
971, Masover et al 1976a, Taylor-Robinson & Carney 197
talheim et al 1976, McGarrity 1985). The data of Figs.
nd 17 show that ureaplasmas also attach to HeLa cells
°C. At 36°C HeLa cells are metabolically active a
nalysis of attachment results may be complicated since t
ool of potential membrane attachment sites may be changi
s a result of membrane turnover. At 4°C this problem do
ot exist because the membrane-turnover does not occ
Anderson & Kaplan 1983, Brunius & Bolin 1983). The He
ell model has been used effectively at 4°C to study t
ttachment characteristics of Yersinia psuedotuberculos
Brunius & Bolin 1983) as well as Chlamydia trachomat
Soderlund & Kilstrom 1983).

The standard reaction volume chosen was 0.2 ml (Fi
6) since this volume minimized the amount of media a
reaplasmas required to achieve a given concentration.

5.3 Methods for detection of attached U. urealyticum

There are few publications that describe methods for detecting ureaplasmas that have attached to eukaryotic cells. The CCU method has been used to monitor the association of U. urealyticum with HeLa (Mazzali & Taylor-Robinson 1971, McGarrity 1985), L132 (Mazzali & Taylor-Robinson 1971), 3T-6 cell cultures (McGarrity 1985) and Fallopian tube organ culture (Taylor-Robinson & Carney 1974). Since the samples enumerated in the present study contained HeLa cells as well as ureaplasmas, I did not want to rely on pH change as an indication of ureaplasma growth, therefore the CFU assay was used. An obvious limitation of the CFU assay is that non-viable ureaplasmas cannot be detected. As such it cannot be used in experiments where the ureaplasmas do not multiply (e.g. exposure to UV light). An assay that was independent of viability was also required. An internal label would have been less likely to interfere with the adhesins on the surface of the ureaplasmas. ^{35}S-methionine labelling of U. urealyticum has been reported by Howard et al (1981). However, I did not obtain adequate specific activity using ^{35}S-methionine or ^{3}H-thymidine, ^{3}H-uracil or a ^{3}H-amino acid mixture. Iodination was rapid and the specific activity was 1 CPM per to 10 ureaplasmas. The ureaplasmas remained viable after iodination. This reflects the mildness of the Iodo-gen method which has already been established for viable eukaryotic cells (Fraker et al 1978). Specificity of the

^{25}I-*U. urealyticum* label was shown by methods similar to those used for studying the attachment of radiolabelled-*M. pneumoniae* to WiDr cells (Chandler *et al* 1984). Fluorescence microscopy, inhibition of binding by unlabelled ureaplasmas, and correlation between CFU and IR assay results support the assumption that the bound radioactivity detected was due to attachment of ^{125}I-*U. urealyticum*. Although unlabelled ureaplasmas decreased the attachment of ^{125}I-*U. urealyticum*, the amount of inhibition was less than expected (Table 10). The ^{125}I-ureaplasmas may be forming clumps which makes inhibition using non-iodinated ureaplasmas less efficient than expected. This explanation seems unlikely since excessive clumps were not observed when ^{125}I-ureaplasmas attached to HeLa cells were observed by FM. Chandler and Barile (1983) reported that inhibition of *M. pneumoniae* attachment to eukaryotic cells could not be achieved when the amount of *M. pneumoniae* was not sufficient to saturate the eukaryotic cell attachment sites. In contrast, competition studies could be readily achieved when the eukaroytic attachment sites were saturated with *M. pneumoniae* (Chandler & Barile 1983). Boatman *et al* (1976) estimated that HeLa cells had a surface area of 886 μm^2 with one face having an area of approximately 443 μm^2. Robertson *et al* (1983) showed by morphometry that, assuming *U. urealyticum* had a spherical shape, then the average cell diameter was 0.5 μm. Based on this information it would be theoretically possible to have approximately 2,000

eaplasmas bound to each cell of the HeLa monolayer. The
nibition of binding with unlabelled ureaplasmas (Table 10)
d not involve sufficient ureaplasmas to saturate all the
tential binding sites on HeLa cells, a possible
planation why it was not possible to inhibit completely
e binding of ^{125}I-U. urealyticum (Table 10).

A limitation of the ^{125}I assay was the rapid
ssociation of the ^{125}I in the presence of serum (Fig. 11).
dioactivity was the only measure used to determine if the
eaplasmas had sedimented. It is possible that some of the
^{5}I detected in the supernatant fluid was due to
sedimented ureaplasmas (Fig. 11). One explaination for
e increase in radioactivity in the supernatant fluid of
e serum treated preparations is that proteases in the
rum may contribute to the removal of the ^{125}I-labelled
mponents. Alternatively the ^{125}I-labelled components of
e ureaplasmas may dissociate and equilibrate with an
abelled component present in the serum. In the presence
serum even the initial readings, taken approximately 5
s after addition of the ^{125}I-U. urealyticum, showed three
six-times more dissociation of ^{125}I than the controls
g. 11). Based on this limitation, all IR assays were
formed using H buffer only. Although surface iodination
frequently used for bacterial attachment studies, little
sideration is given to the dissociation of the labelled
mponents. This phenomenon is not unique to ureaplasmas;
gasawara et al (1983) noted a similar decrease in cell

ramine-T method) was incubated in ascites fluid, which is rous fluid.

Since the average cell diameter of a ureaplasma cell .5 µm (Robertson et al 1983) it is difficult to monitor achment by light microscopy unless special stains are l. Shepard (1957) used Giemsa stained preparations in junction with light microscopy, to examine "PPLO-like" ls in urethral scrapings. However, the Giemsa stain ks specificity. Indirect immunofluorescence has been d to identify mycoplasmas attached to eukaryotic cells tman et al 1976, Butler & Stanbridge 1983). The same ic method is also applied in the colony-fluorescence typing scheme for ureaplasmas (Black & Krogsgaard-Jensen , Robertson & Stemke 1982). Fluorescent ureaplasmas readily visualized using our FM procedures. Background rescence was low in the negative controls (Figs. 10 &

Because of the difficulty in accurately counting the rescent particles, FM was used primarily as visual irmation of ureaplasma attachment to HeLa cells by the and IR assays.

The HeLa 229 cell model described here was useful for dying attachment characteristics but not for studying onic infection. The FM and IR detection methods mented the CFU assay. The CFU method proved a reliable bility assay and the IR method a useful alternative for dying the attachment of non-viable ureaplasmas to aryotic cells.

. Introduction

Despite the established importance of the attachment of prokaryotes in the disease process, there has been little progress in studying the attachment of ureaplasmas to eukaryotic cells. The results presented in Ch. IV demonstrated that U. urealyticum strain 27 attached to HeLa cells. My next objective was to characterize more completely the attachment process. Scanning and transmission electron microscopy studies were designed to determine if ureaplasmas could be observed on the surface of infected HeLa cells. Some of the attachment characteristics of ureaplasmas were investigated using the CFU and IR assays that were described in Ch. IV. The ureaplasma membrane was studied to identify those components that might be involved in attachment.

. Microscopy of U. urealyticum cells attached to HeLa monolayers.

Ureaplasma-infected and ureaplasma-free HeLa monolayers were prepared for scanning electron microscopy (SEM) by the method described in Appendix II, Sect. 2. The morphology of ureaplasma-free HeLa cells (Fig. 18) appeared similar to the that described for normal HeLa cells (Rose 1970). Mycoplasma-like forms, i.e. spheres of about 0.5 to 1 μm diameter, were observed on the infected monolayers (Figs.

9 & 20) but not on the control monolayers (Fig. 18). FM of replicate coverslips confirmed that ureaplasmas could be detected only on infected monolayers (Fig. 21). By both FM and SEM the number of ureaplasmas apparent on the surface of the HeLa cells varied from only a few to several hundred. Long microvilli were observed more frequently on infected monolayers (Fig. 20) than on the corresponding control monolayers that were ureaplasma-free (Fig. 18). TEM of ureaplasma infected HeLa cells suggested that the ureaplasmas were extracellular (Fig. 22). Occasionally, ureaplasmas that were closely associated with the HeLa cell membrane were apparent in TEM (Fig. 22B). The gap between the opposing ureaplasma and HeLa membranes, shown in the insert, is approximately 10 nm to 15 nm (Fig. 22B).

3. Attachment of U. urealyticum strain 27 to HeLa 229 cells.

3.1 Inoculum

Increasing concentrations of ^{125}I-U. urealyticum were added to HeLa monolayers to determine if attachment was concentration dependent. There was a linear relationship between the amount of ^{125}I-ureaplasmas added and the amount of radioactivity that bound to the HeLa cells (Fig. 23).

3.2 Viability

If the components involved in ureaplasma attachment need to be actively synthesized then non-viable ureaplasmas would not attach. To determine the effect of viability on attachment, ureaplasmas were exposed to ultra-violet light

All of the HeLa cell monolayers used for SEM were
plicates. The uninfected monolayer was incubated and
epared for SEM as described in Appendix II, Sect. 2.
ort microvilli, indicated by asterisks, were the major
rface features noted in this negative control. The
gnification is x 20,875. The bar represents 1 μm.

monolayers infected with Ureaplasma urealyticum strain 27.

The HeLa cell monolayer was a replicate of that prepared in Fig. 18. The inoculum was 10^8 CCU of ureaplasmas per HeLa monolayer. The monolayers were incubated at 36°C for 6 hrs prior to processing for SEM (Appendix II Sect. 2). The large number of ureaplasmas that could attach per HeLa cell is shown in (A). Arrows indicate some of the ureaplasmas. The magnification is x4,400. The bar represents 10 μm. (B) is an enlargement of (A) and shows more clearly some of the ureaplasmas attached to eukaryotic cell projections. The magnification is x19,500. The bar represents 1 μm.

A
B

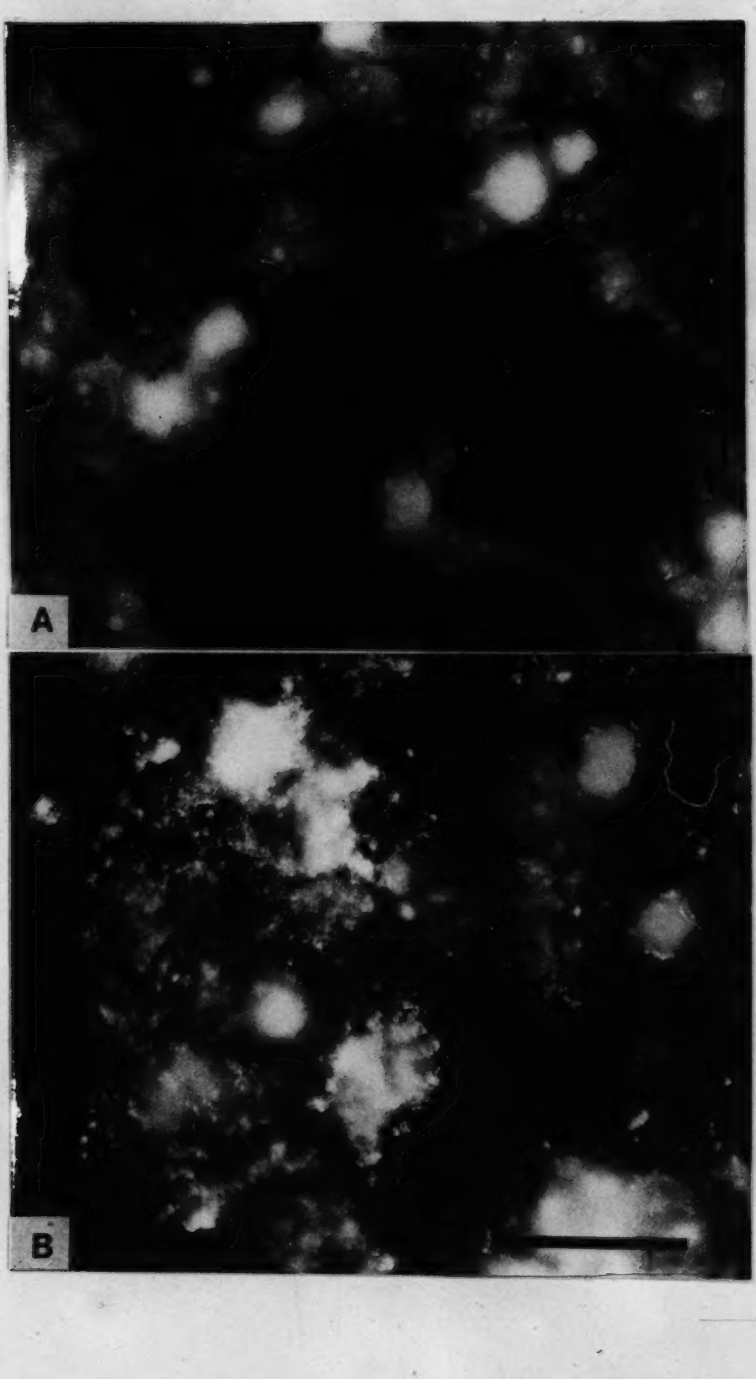
Figure 20. Scanning electron micrographs of HeLa 229 cells infected with Ureaplasma urealyticum strain 27; association with HeLa microvilli.

These monolayers were replicates of those prepared in Fig. 19. Asterisks indicate the long HeLa microvilli and arrows indicate the ureaplasmas (A). The magnification is x9,428. The bar represents 1 μm. (B) is a higher magnification of (A) and shows some ureaplasmas that are closely associated with the microvilli. The magnification is x16,500. The bar represents 1 μm.

A
B

Ureaplasma urealyticum strain 27 and observed by fluorescence microscopy.

These monolayers were replicates of those used for SEM (Figs. 19 & 20). After fixation and application of the primary antibody used in SEM (Appendix II Sect. 2), the usual FM procedures were used (Ch. II, Sect. 7.2). The ureaplasma-free control is shown in (A). The background fluorescence of the HeLa cells is more than normally observed in FM and is due to autofluorescence of proteins that have been treated with glutaraldehyde. (B), shows monolayers that were infected with U. urealyticum. The arrows indicate fluorescent particles that are of appropriate dimensions to be individual ureaplasmas. The magnification is x500. The bar represents 50 μm.

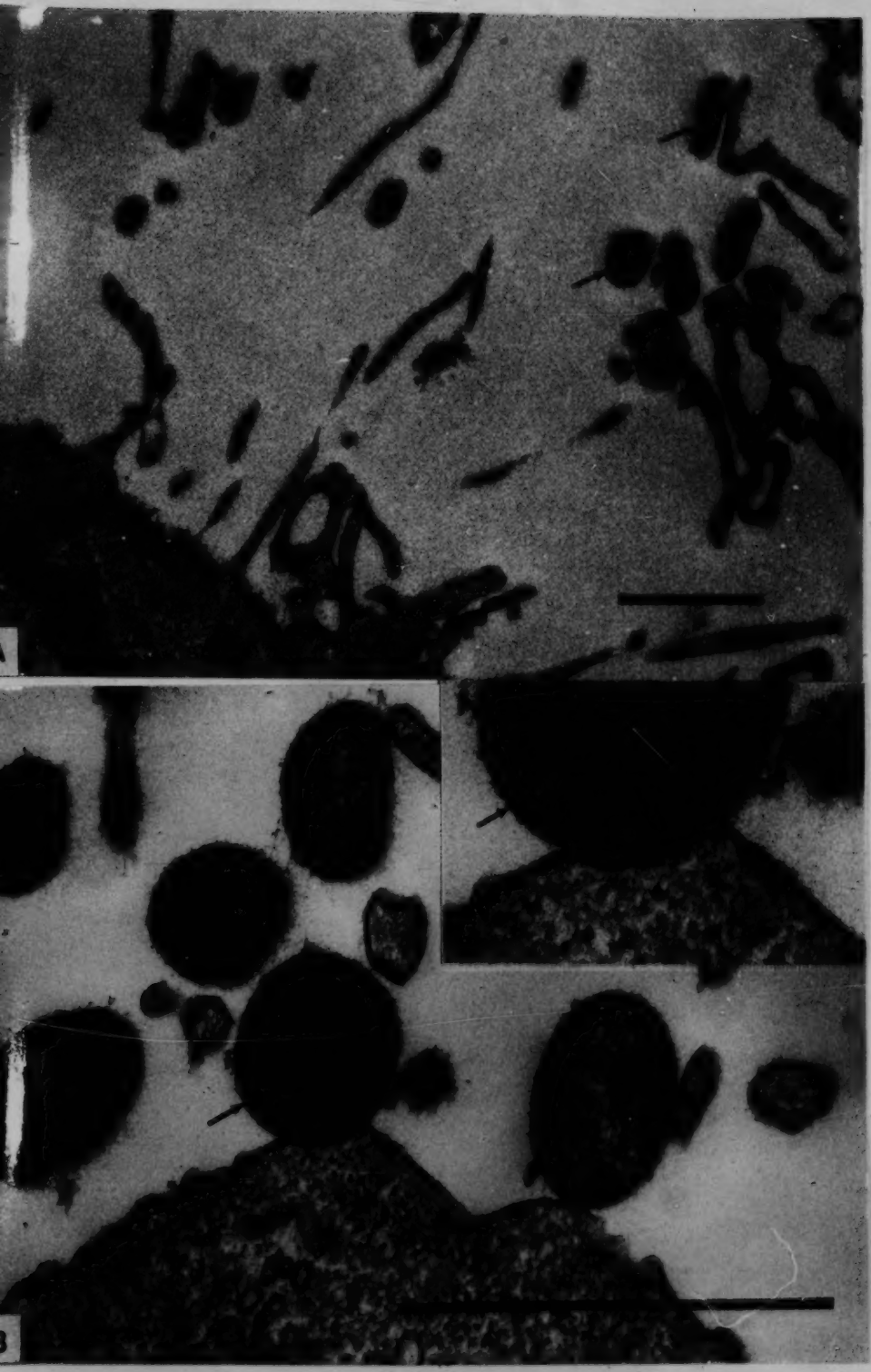

A
B

igure 22 Transmission electron microg
cells infected with Ureapl
strain 27.

The inoculum was 10^7 CCU ureap
onolayer. The monolayers were incubated a
rior to processing for TEM (Append
requently, ureaplasmas were apparent near
f the HeLa cells (A). The HeLa cell
ndicated by asterisks and the ureaplasmas
rrows. The magnification is x24,000. Th
μm. Less frequently, ureaplasmas were c
ith the HeLa cell membrane as shown in
e indicated by arrows. The magnificatio
ar represents 1 μm. The inset is an en
reaplasma/HeLa cell membrane interface s
gnification of the inset is x144,000.

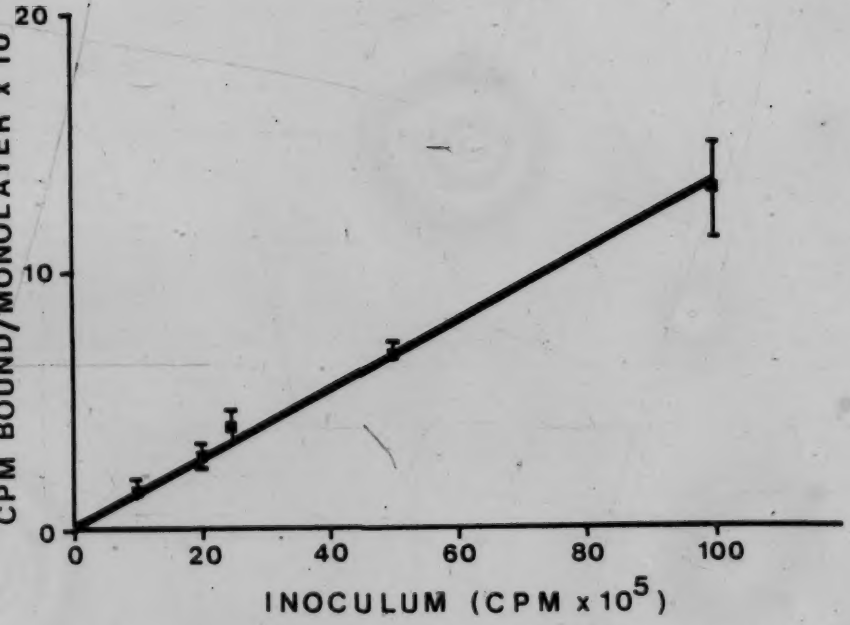
raphs of HeLa 229
asma urealyticum

lasmas per HeLa
at 36°C for 24 hrs
ix II Sect. 1).
the microvilli
microvilli are
are indicated by
he bar represents
losely associated
(B). Ureaplasmas
n is x72,000. The
nlargement of the
hown in (B). The



ure 23 The effect of varying the inoculum on the attachment of ^{125}I-*Ureaplasma urealyticum* strain 27 to HeLa 229 cells.

The monolayers were inoculated with varying centrations of ^{125}I-ureaplasmas (specific activity of 1.4 per ureaplasma) and then incubated at 4°C for 1 hr. The assay was used to determine the amount of radioactivity t bound per monolayer (Ch. II, Sect. 7.8.3). Each CPM lue is the mean ± the standard deviation of duplicate terminations. The data were analyzed using linear ression and the r^2 value was 0.99.


CPM BOUND/MONOLAYER x 10
20
10
0
0
20
40
60
80
100
INOCULUM (CPM x 10^5)

(Ch. II, Sect. 7.3) which denatures the DNA and prevents the ureaplasmas from multiplying. HeLa cells were fixed with glutaraldehyde. Neither of these treatments abolished binding completely but the number of ureaplasmas that attached was about half that observed for the control (Table 13). Fluorescence microscopy confirmed the CFU and IR assay results.

3.3 Medium components

Ureaplasmas are fastidious, with urea and serum supplementation among their nutrient requirements for *in vitro* growth. These microorganisms are known to adsorb components from the growth medium. Urea, BSA, and cholesterol, which may be components of the ureaplasma growth media, were tested to determine if they had any effect on the attachment of ureaplasmas to HeLa cells (Table 14). As expected, the viability of *U. urealyticum* was not affected by any of these components at the concentrations listed in Table 14. For all concentrations of the urea and BSA that were tested, the mean CFU bound per monolayer was within two standard deviations of the control mean (Table 14). The presence of 5 or 0.5 mM cholesterol resulted in binding that was significantly less than the control mean (Table 14). However, when 0.05 mM cholesterol was added, no significant decrease in the attachment of ureaplasmas was observed.

3.4 Antisera

If attachment of a pathogenic microorganism can be

Table 13 The effect of viability on the attachment of *Ureaplasma urealyticum* strain 27 to HeLa 229 cells.

Treatment	Ureaplasma Inoculum/ monolayer (SD*)	Ureaplasmas Bound/ monolayer (SD*)	% bound
	CPM	CPM	
UV treated** ureaplasmas	6.2×10^4 (.88)	4.5×10^2 (.48)	0.7
Control+	1.7×10^5 (.15)	2.9×10^3 (.46)	1.7
	CFU	CFU	
Glutaraldehyde*** fixed HeLa cells	3.1×10^5 (1.60)	2.9×10^3 (.39)	0.9
Control+	3.2×10^5 (1.10)	7.9×10^3 (3.2)	2.5

* SD denotes standard deviation.

** The ureaplasmas were pre-treated by U.V. irradiation (Ch. II, Sect. 7.3). The IR assay was used for this experiment (Ch. II, Sect. 7.8.3). Each CPM value was the mean ± SD of four replicate determinations.

*** The HeLa cell monolayers were treated with 0.05% (w/v) glutaraldehyde as described in Ch. II, Sect. 6.2. Ureaplasmas were then inoculated and a standard CFU assay performed (Ch. II, Sect. 7.8.2). Each CFU value was the mean ± SD of four replicate determinations.

+ The controls consisted of viable ureaplasmas and viable HeLa cells.

Table 14 The effect of urea, bovine serum albumin and cholesterol on the attachment of U. urealyticum strain 27 to HeLa 229 cells.

Component* added (mM)	Inoculum/ monolayer CFU x 10^7	(SD^+)	Bound/ monolayer CFU x 10^6	(SD^+)	(%)
Urea:					
10.00	2.7**	(.42)	1.5	(.42)	5.5
2.50	2.7	(.32)	1.9	(.69)	7.0
0.25++	2.6	(.23)	1.9	(.25)	7.3
BSA:					
10.0	2.9	(.61)	2.0	(.16)	6.9
1.0++	2.8	(.59)	1.7	(.31)	6.1
0.1	2.7	(.51)	1.6	(.37)	5.9
Cholesterol:					
5.00	2.5	(.48)	0.46#	(.06)	1.8
0.50	2.4	(.24)	0.43#	(.12)	1.6
0.05++	2.4	(.15)	0.99	(.26)	4.1
H buffer (control)	2.2	(.10)	1.80	(.20)	8.2

* All components were prepared in H buffer and then the number of ureaplasmas indicated was inoculated.

+ SD denotes standard deviation.

** Standard CFU assay conditions (Ch. II, Sect. 7.8.2) were used. Each CFU value was the mean ± SD of four replicate determinations.

++ This is the concentration routinely incorporated into B broth and GM agar.

Using the two tailed Student t test at the 0.01 level of significance, this mean was significantly different than the control of 1.80 x 10^6 CFU bound per monolayer.

inhibited by antibodies then immunization might prove useful as a means of preventing disease. Experiments were devised to test the effect of polyclonal antisera directed against whole ureaplasmas as well as polyclonal antiserum directed against horse serum which ureaplasmas adsorb from the growth medium. The CFU assay was used to monitor the effect of antiserum on attachment. None of the antisera tested affected the viability of U. urealyticum when held at 4°C for 1 hr. The attachment of U. urealyticum strain 27 to HeLa cells was not altered by antisera to the homologous or heterologous strain (Table 15). For all of the sera tested, the mean CFU bound per monolayer was within two standard deviations of the control mean (Table 15).

4. Determination of the membrane components of U. urealyticum strain 27

The following studies were undertaken to identify ureaplasma surface components which may be involved in attachment to host tissues. ^{125}I-U. urealyticum whole cell extracts were subjected to SDS-PAGE to determine which components were iodinated (Fig. 24). The bands on the SDS-PAGE were transferred electrophoretically to nitrocellulose (Ch. II, Sect. 4.2). To differentiate those components that were produced by U. urealyticum from those that were derived from horse serum, the banding patterns on separate nitrocellulose sheets were tested with rabbit anti-horse serum and rabbit anti-U. urealyticum strain 27 serum (Fig. 24). The two heavily iodinated areas of high molecular

Table 15 The effect of normal and immune rabbit sera on the attachment of Ureaplasma urealyticum strain 27 to HeLa 229 cells.

Rabbit* serum added (% vol/vol)	Inoculum/ monolayer		Bound/ monolayer		
	CFU x 10^7	(SD^+)	CFU x 10^6	(SD^+)	(%)
normal:					
0.1	3.0	(.37)	1.7	(.52)	5.7
0.01	3.0	(.41)	1.1	(.30)	3.7
anti-horse:					
0.1	3.0	(.50)	1.0	(.34)	3.3
0.01	3.0	(.39)	1.2	(.34)	4.0
anti-U. urealyticum strain T960-CX8:					
0.1	2.7	(.22)	1.2	(.68)	4.4
0.01	3.1	(.27)	0.8	(.16)	2.6
anti-U. urealyticum strain 27:					
0.1	2.9	(.37)	1.5	(.17)	5.2
0.01	2.9	(.36)	1.7	(.17)	5.9
H buffer (no serum)	3.1**	(.54)	1.2	(.35)	3.9

* All attachment assays were performed using H buffer with the amount of added serum indicated, and using standard CFU assay conditions (Ch. II, Sect. 7.8.2).

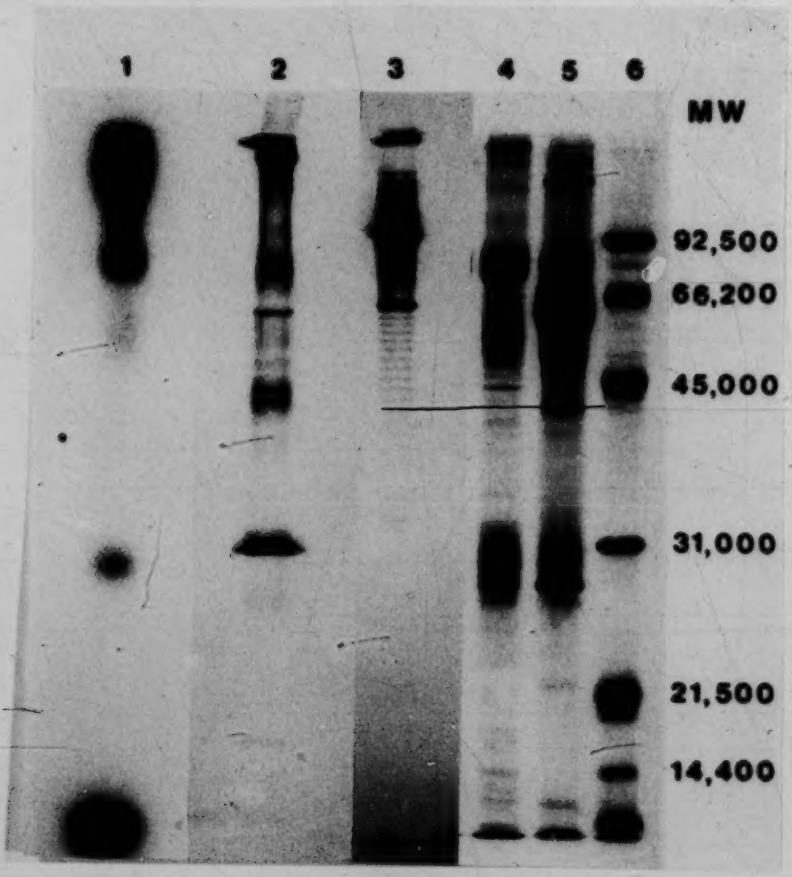
+ SD denotes standard deviation.

** Each CFU value was the mean ± SD of four replicate determinations.

^{125}I-*Ureaplasma urealyticum* strain 27.

All samples for SDS-PAGE were unreduced and were electrophoresed on a 12.5% polyacrylamide gel (Ch. II, Sect. 4.1). This is a composite figure therefore migration distances may not be exactly the same in each lane.

Lane number	Components/Treatment
1	Autoradiogram of SDS-PAGE a whole cell extract of ^{125}I-*U. urealyticum*.
2	Immunoblot of SDS-PAGE of a whole cell extract of ureaplasmas. The primary antiserum was rabbit anti-horse serum. The immunoblotting method was described in Ch. II, Sect. 4.2.
3	Immunoblot of SDS-PAGE of a whole cell extract of ureaplasmas. The primary antiserum was rabbit anti- *U. urealyticum* strain 27 serum.
4	SDS-PAGE of a whole cell extract of ureaplasmas, stained with Coomassie blue.
5	SDS-PAGE of unconcentrated B broth, stained with Coomassie blue.
6	SDS-PAGE of molecular weight markers, stained with Coomassie blue.


1
2
3
4
5
6
MW
92,500
66,200
45,000
31,000
21,500
14,400

weight material seen in Fig. 24, lane 1 correspond to areas that reacted with anti-horse serum antibodies (Fig. 24, lane 2).

Although serum is an important nutritional factor, little is known about the role, if any, of serum in attachment. Since attachment is mediated by surface structures, the possible role of horse serum components adsorbed onto the surface of ureaplasmas was examined. The results (Fig. 24) indicate that despite six washings of the ureaplasma cells, horse serum components were still present. The following experiment was designed to determine how readily serum components could be removed and the effect this had on the attachment of ureaplasmas to HeLa cells. Since most of the ^{125}I had labelled components that reacted with anti-horse serum antibodies, the dissociation of ^{125}I from ^{125}I-ureaplasmas was monitored as an indication of the amount of horse serum that was removed. It is unlikely that the ^{125}I-labelled components are integral proteins since they were dissociated in a dose-dependent manner by serum (Fig. 11). Based on the premise that the adsorbed serum components were likely to be peripheral proteins, the dissociation of ^{125}I was examined in the presence of agents that would be expected to remove peripheral proteins (Table 16). The agents tested were EDTA (a chelating agent), trypsin (a proteolytic enzyme), urea (a disrupter of hydrophobic bonds), guanidine thiocyanate (a chaotropic agent), cholesterol (a component known to be adsorbed from

Table 16 The effect of ^{125}I dissociation from Ureaplasma urealyticum strain 27 on viability and attachment to HeLa 229 cells.

Treatment*	Viability CCU**	^{125}I dissociated (%)	CPM+ bound/monolayer (%)
EDTA (10 mM)	10^5	6	5
Cholesterol (2.6 mM)	10^6	6	5
Horse serum (10 %)	10^6	28	5
Trypsin (0.25 %)	10^4	32	2
Urea (8 M)	10^3	45	3
Guanidine thiocyanate (6 M)	0	81	2
Control (H buffer)	10^6	7	5

* All components were dissolved in 1 ml H buffer pH 6.0 and then 9.9 x 10^6 CPM of ^{125}I-U. urealyticum strain 27 (specific activity of 9.9 CPM per CCU of ureaplasmas) was added. The suspensions were incubated at RT for 15 min. and then the ureaplasma cells were collected by centrifugation at 15,000 rpm for 5 min. The amount of dissociated ^{125}I in the supernatant fluids was measured.

** The CCU estimates were results of single tests on the ureaplasma cell pellets after each treatment.

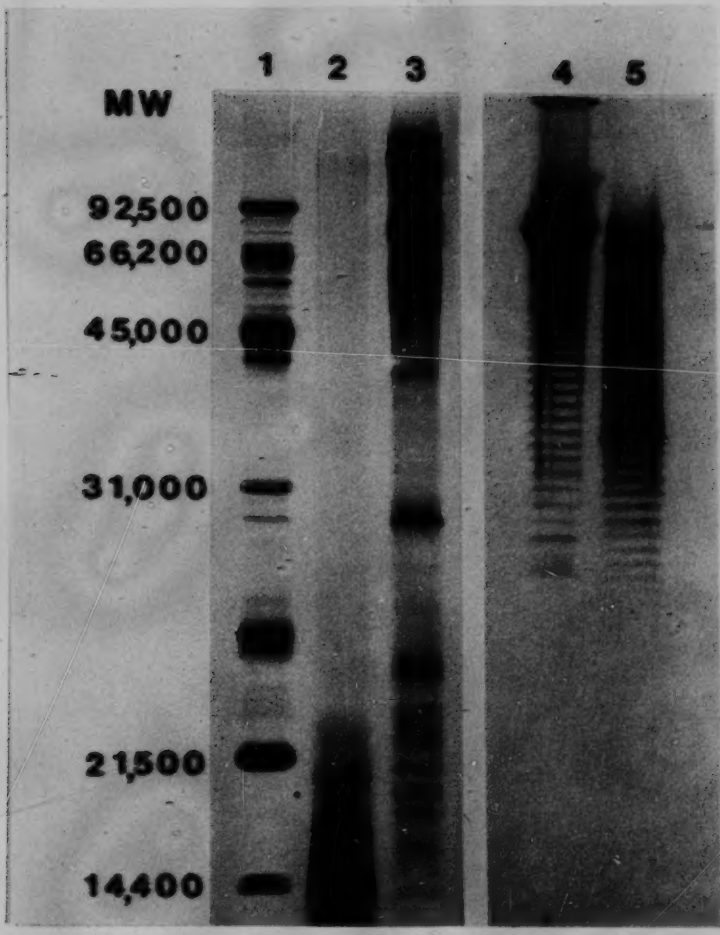
+ The ureaplasma cell pellets were resuspended in H buffer and used for standard IR attachment assays (Ch. II, Sect. 7.8.3). Each value was the mean of duplicate determinations and has been expressed as the nearest whole number.

exposed to each treatment then tested for viability, the amount of ^{125}I that had dissociated, and attachment to HeLa cells (Table 16). Horse serum was the only treatment that caused significant dissociation of ^{125}I from ^{125}I-ureaplasmas but did not affect viability or attachment to HeLa cells (Table 16). The proteolytic enzyme trypsin removed ^{125}I but attachment was also significantly decreased. Further studies were carried out to determine the effects of protease treatment on the SDS-PAGE patterns of ^{125}I-ureaplasmas. No high molecular weight material was observed when SDS-PAGE gels of proteinase K treated ureaplasmas were silver-stained (Figure 25). However, when proteinase K digested material was transferred electrophoretically from the polyacrylamide gel to nitrocellulose and probed with rabbit anti-*U. urealyticum* serum, bands were detected (Fig. 25, lanes 4 & 5) that were reminiscent of the "ladder patterns" produced by the lipopolysaccharide of Gram-negative bacteria (Mintz *et al* 1984). The proteinase K digested material was dialysed exhaustively against distilled water and then subjected to gas liquid chromatography (Fig. 26). When this preliminary chromatogram was compared to carbohydrate standards, the proteinase K resistant material contained components that eluted at the same time as glucose and mannose (Fig. 26).

gure 25 Immunoblot and silver stain of SDS-PAGE of a proteinase-K treated, whole-cell extract of Ureaplasma urealyticum strain 27.

All samples for SDS-PAGE were unreduced and were ectrophoresed on a 14% polyacrylamide gel (Ch. II, Sect. 1). This is a composite figure therefore migration stances may not be exactly the same in each lane.

Lane	Component/Treatment
1	SDS-PAGE of molecular weight markers that were stained with silver.
2	SDS-PAGE of a proteinase K treated, whole-cell extract of U. urealyticum that was stained with silver.
3	SDS-PAGE of a whole-cell extract of U. urealyticum that was stained with silver.
4	Immunoblot of SDS-PAGE of a whole-cell extract of U. urealyticum. The primary antiserum was rabbit anti-U. urealyticum strain 27 serum. The immunoblotting method was that described in Ch. II, Sect. 4.2.
5	Immunoblot of SDS-PAGE of a proteinase K treated whole- cell extract of U. urealyticum. The primary antiserum was rabbit anti-U. urealyticum strain 27 serum.

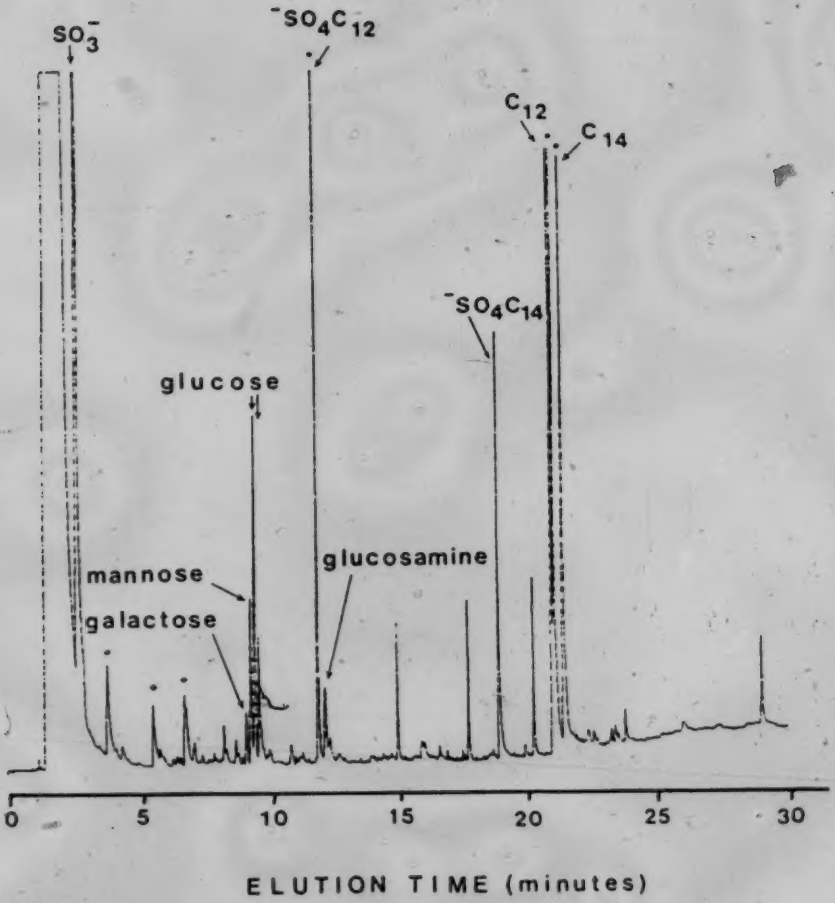

1
2
3
4
5
MW
92,500
66,200
45,000
31,000
21,500
14,400

igure 26 Gas chromatogram of a whole-cell digest of Ur strain 27.

The proteinase K digest pr reaplasmas (Ch. II, Sect 7.6.1) was nd then approximately 1 mg wa hromatography as described in Ch. onditions outlined in Ch. II, Sect. as chromatography. A control of Lae uffer (Appendix I, Sect. 3.1.3) wa anner. The asterisks on the chroma eaks that were also observed on the ample buffer alone. Methanolysis of auryl cleaves SO_4 from the carbon c artial under mild conditions. . urealyticum sample were iden etention times of the unknown peaks f the standards for galactose, lucosamine, SO_3^-, $^-SO_4C_{12}$, $^-SO_4C_1$ hemical Co.).

proteinase K treated,
reaplasma urealyticum

epared from 1 L of
dialyzed, lyophylized
as prepared for gas
II Sect. 7.6.2. The
7.6.2 were employed for
mmli's modified sample
s treated in the same
togram indicate those
control chromatogram of
sodium dodecyl sulfate
chain. The reaction is
The peaks in the
ntified by comparing
to the retention times
mannose, glucose,
$_4$, C_{12} and C_{14} (Sigma


SO_3^-
$^-SO_4C_{12}$
C_{12}
C_{14}
$^-SO_4C_{14}$
glucose
mannose
galactose
glucosamine
0
5
10
15
20
25
30
ELUTION TIME (minutes)

5. Discussion

5.1 Visualization of ureaplasmas attached to the eukaryotic cells.

Although it proved invaluable for visualizing ureaplasmas attached to HeLa monolayers, FM does not provide detailed information regarding the attachment of ureaplasmas to HeLa cells. SEM and TEM of ureaplasma infected HeLa monolayers allowed more detailed examination of the morphological characteristics of the ureaplasmas that had attached to HeLa cells. To my knowledge, Figs. 19 and 20 are the first SEM demonstrating ureaplasmas associated with HeLa cells. Approximately 1,000 times more ureaplasmas were inoculated for SEM than were inoculated for attachment assays. The larger inoculum was necessary to differentiate between ureaplasma-infected and uninfected HeLa cells. Others have also reported that when only a few mycoplasmas were attached then it was difficult to differentiate mycoplasmas from microvilli or other eukaryotic surface structures (Jaunzems & Ellis 1982, McGarrity 1982). The ureaplasmas did not uniformly cover the HeLa cells surface (Fig. 20A). I observed some cells having only about 10 to 50 ureaplasmas (Fig. 20A) while others had several hundred (Fig. 19A). This uneven distribution of ureaplasmas was also apparent when infected monolayers were observed by FM (Fig. 10). McGarrity (1985) also noted that ureaplasmas formed microcolonies on some but not all HeLa cells. It is possible that ureaplasmas preferentially attach to HeLa

cells at specific stages in the eukaryotic cell cycle. The HeLa cells used in these experiments had all been incubated for 18 hrs to 24 hrs at 36°C and since the cultures were not synchronized there would be cells in various stages of the growth cycle. Synchronization of the HeLa cells would be necessary to determine if ureaplasmas attach preferentially to HeLa cells in a specific stage of the growth cycle. The ureaplasma cells that I observed were predominantly uniform spheres (Figs. 19 & 20), similar to the morphology reported for other mycoplasmas (Boatman et al 1976, Jaunzems & Ellis 1982, McGarrity et al 1984). HeLa cells heavily infected with ureaplasmas had more long cytoplasmic processes (Fig. 19) than did the negative controls (Fig. 18) that had been prepared and incubated under identical conditions. This is consistent with reports for other mycoplasmas (Zucker-Franklin et al 1966, Stanbridge 1971, Boatman et al 1976). Zucker-Franklin et al (1971) suggested that the development of the many cytoplasmic processes may be a means whereby the infected eukaryotic cell attempts to compensate for the surface area occupied by the mycoplasmas.

To ensure that many ureaplasmas would be bound per HeLa cell a large number of ureaplasmas (10^7 CCU to 10^8 CCU) were used to inoculate HeLa cells that were used for TEM. The ureaplasmas observed by TEM were more electron dense than the eukaryotic cell structures (Fig. 22). The morphology of the ureaplasmas observed in TEM of infected HeLa cells was similar to that reported previously for ureaplasmas and also

for other mycoplasmas (Zucker-Franklin et al 1966, Black et al 1972, Boatman et al 1976). In most of the TEM the ureaplasmas were about 10 to 500 nm from the eukaryotic microvilli (Fig. 22A). When ureaplasmas were observed close to the eukaryotic cell surface (Fig. 22B), the gap between the opposing ureaplasma and eukaryotic membranes was about 10 to 15 nm. This distance is similar to that reported for the Mycoplasma sp. studied by Boatman et al (1976). Such a gap is too large to allow direct bridging (a Ca^{+} bridge between two carboxyl groups requires a 1 nm gap (Watt et al 1978)). The ureaplasmas "capsule" reported by Robertson and Smook (1976) is 20 nm to 50 nm wide. The glycocalyx of eukaryotic cells is approximately 50 nm thick (Alberts et al 1983). It is possible that the 10 nm to 15 nm gap observed in Fig. 22B is the overlapping area of the HeLa cell glycocalyx and the ureaplasma capsule. Even when observed 48 hrs to 72 hrs post-infection, no ureaplasmas were apparent inside the HeLa cells. Although SEM and TEM provide more detailed information than FM, a specific ureaplasma label is needed so that the ureaplasma cells can be differentiated conclusively from the eukaryotic components.

5.2 Characteristics of attachment

The binding of U. urealyticum to HeLa cells at 4°C and 36°C was time-dependent (Figs. 9, 10, 11 & 14). Yet after 6 hrs of contact only 5% to 10% of the inoculated ureaplasmas were attached. These results suggest that only a small

portion of ureaplasma cells are capable of binding or that only a small fraction of the cells come in contact with the HeLa cells. Similarly, binding of approximately 10% of the inoculum has been reported for Chlamydia trachomatis and Mycoplasma pneumonia (Soderlund & Khilstrom 1983, Chandler et al 1984).

The relationship between the concentration of ^{125}I-ureaplasmas inoculated and the amount of radioactivity that bound per monolayer (Fig. 23) was not directly proportional. Attachment of ureaplasmas at one site may not be independent of other attached ureaplasmas or repulsion may be possible explainations. Under the conditions of the experiment saturation of the HeLa cells with ureaplasmas was not possible. Saturation of confluent WiDr cells with approximately 40 µg of M. pneumoniae protein was reported by Chandler et al 1984, but no indication was given of the number of viable cells this represented. Boatman et al (1976) used morphometric analyses to show that HeLa cells infected with bovine Mycoplasma sp. carried an average of 69 mycoplasmas per cell. As discussed in Ch. IV it is theoretically possible to have about 2,000 ureaplasmas bound per HeLa cell. The SEM results presented in this chapter (Figs. 19 & 20) demonstrated that hundreds of ureaplasmas could associate with a single HeLa cell. Based on this information, the inocula of about 10^5 CCU per monolayer used in my attachment studies would not be sufficient to obtain saturation of the HeLa binding sites. To obtain saturation of binding sites in this model (i.e. where there

were 10^5 HeLa cells per monolayer), the ratio of ureaplasmas to HeLa cells should be at least 10,000 to 1 (i.e. about 1 L of harvested ureaplasmas per monolayer). Rather than increasing the inoculum or incubation time, an alternative would be to modify the conditions used in this study and grow the monolayers in smaller wells, thereby decreasing the HeLa cell number. The ratio of 10,000 ureaplasmas to 1 HeLa cell could be achieved using fewer ureaplasmas. This approach has been successfully used by Chandler and Barile (1983) to saturate the attachment sites on WiDr cells with M. pneumoniae. Saturation of binding sites will be necessary for analysis of ureaplasma attachment kinetics. The present studies were designed to develop a model and reliable assays to begin characterizing attachment of ureaplasmas to eukaryotic cells.

Attachment of UV treated ureaplasmas to HeLa cells was approximately one-half that observed for controls (Table 13). Ureaplasmas do not proliferate when incubated at 4°C yet they are still able to attach to HeLa cells (Fig. 23). These results indicate that active proliferation is not necessary for attachment of ureaplasmas to eukaryotic cells. Similar results have been reported for other mycoplasmas. Banai et al (1981) reported that UV irradiation effectively killed M. gallisepticum but did not completely inhibit attachment of M. gallisepticum to eukaryotic cells.

5.3 Possible components involved in attachment.

By definition, the components involved in adhesin-receptor reactions must be surface components oriented in a

manner that allows interaction (Beachey 1981). Attachment between the microorganism and the eukaryotic cell can occur by stereo-specific, hydrophobic, or ionic interactions. Competitive inhibition assays are frequently used to determine components that are involved in stereo-specific attachment. Inhibition of attachment can be achieved using unlabelled microorganisms, purified adhesin or receptor components (or their analogues), antibodies, or lectins (Beachey 1981, Chandler et al 1984). Potential inhibitors of attachment were tested to identify which factors may be involved in ureaplasma attachment to HeLa cells. Also, preliminary work was initiated to identify those components that were located on the outer membrane surface of ureaplasmas.

5.3.1 Potential inhibitors of attachment.

Urea is necessary for growth of ureaplasmas, and cholesterol is adsorbed from serum directly into the ureaplasma membrane. The addition of up to 10 mg/ml (160 mM) urea (i.e. forty-times the concentration of urea normally found in B broth) or up to 10 mg/ml BSA (i.e. twenty-times the concentration normally found in B broth) did not alter the attachment of ureaplasma cells at 4°C (Table 14). Preliminary results indicated that cholesterol may decrease binding of ureaplasmas (Table 14). The concentrations of cholesterol which significantly inhibited binding (0.5 and 5 mg/ml) (Table 14) were about ten to one hundred-times more than that normally found in

B broth. This could mean that cholesterol or other lipids may be involved in either stereo-specific or hydrophobic interactions between the ureaplasma and eukaryotic cell membranes.

5.3.2 Antisera

The antisera used in this study had been raised to whole cell suspensions of ureaplasmas grown in B broth supplemented with rabbit serum (Ch. II, Sect. 5). Thus, this polyclonal serum should contain antibodies directed predominantly against ureaplasma encoded components. The ureaplasmas used for attachment studies were grown in broth supplemented with horse serum. Therefore, anti-U. urealyticum strain 27 serum would not be expected to react with the adsorbed horse serum components present in the ureaplasma membrane. Polyclonal antiserum raised against horse serum was used to determine whether antibodies to the horse serum components of the ureaplasma membrane could inhibit attachment and, in this way, establish if horse serum components were involved in attachment. None of the sera tested altered the viability of U. urealyticum strain 27 incubated at 4°C for up to 4 hrs. Attachment of ureaplasmas to HeLa cells was not altered by either the normal or the immune sera tested (Table 15). The antiserum raised against horse serum and the anti-U. urealyticum strain 27 serum may be unable to prevent attachment because the ureaplasma adhesins were non-immunogenic, the horse serum components on the surface of

the ureaplasmas were not involved in binding, or the ureaplasma adhesins were masked by either ureaplasma proteins that were not involved in attachment, or by adsorbed horse serum components. Alternatively, stereo-specific interactions may not be involved. If hydrophobic interactions are important then a large amount of immunoglobulin would be required to completely coat the ureaplasma before any inhibition would be observed. The conditions used may have been inadequate for antibodies to interact completely with all available epitopes and incubation at 36°C may be necessary to achieve inhibition. However, at 36°C, in the presence of antiserum directed against either U. urealyticum (Lin & Kass, 1970) or horse serum (Sethi & Brandis 1972), immune inactivation occurs. The use of purified immunoglobulins rather than whole antiserum should facilitate the study of the effect of antiserum on the attachment of ureaplasmas to eukaryotic cells at 36°C.

5.3.3 Surface components of U. urealyticum

Previous studies did not determine whether the observed carbohydrate was produced by the ureaplasmas or was adsorbed from the growth medium (Whitescarver et al 1975, Robertson & Smook 1976, and Smith 1984a). Carbohydrates are important mediators of attachment for other microorganisms. The carbohydrate-like material (Fig. 26) that was extracted from U. urealyticum strain 27 was immunogenic (Fig. 25) indicating that it was produced by the ureaplasmas, but

exposure to homologous antiserum did not inhibit binding (Table 15). This indicates that either the carbohydrate material is masked, thereby preventing the antibodies from attaching, or that it is not involved in attachment.

At least two of the bands on the immunoblots of washed U. urealyticum strain 27 cells that reacted with antiserum against horse serum (Fig. 24) corresponded to bands that were ^{125}I-labelled. This suggests that the adsorbed horse serum components of the ureaplasma membrane have an appropriate orientation to function as adhesins. Serum is known to contain components such as fibronectin that are important in attachment of other microorganisms (Beachey et al 1983). Based on the known importance of serum components in other systems and the orientation of serum components on the surface of the ureaplasmas, I attempted to determine how easily serum could be removed from the ureaplasma membrane and what effect removal of the serum components had on attachment. The immunoblots (Fig. 26) indicate that even after multiple washes in H buffer, serum remains associated with the ureaplasma membrane. The serum components of washed ureaplasma membranes were radio-labelled when whole cell suspensions were iodinated. Of the six treatments tried, only horse serum could remove the ^{125}I-components without altering either viability or attachment to HeLa cells (Table 16). The dissociation occurred rapidly; when ^{125}I-ureaplasmas were held in 10% horse serum at 4^{o}C, approximately 30% of the ^{125}I dissociated in 15 mins.

Analysis of the dissociated material by SDS-PAGE, showed that discrete bands of radiolabelled material were present. It seems likely that the serum had displaced rather than destroyed the ^{125}I-labelled components since neither viability nor attachment to HeLa cells was altered. While cholesterol is known to be adsorbed from horse serum directly into the U. urealyticum membrane, the dissociation of ^{125}I in the presence of cholesterol (1 mg/ml) was similar to that observed for the control (Table 16). Although cholesterol could inhibit attachment of ureaplasmas to eukaryotic cells (Table 14), once the cholesterol was removed no inhibition of ureaplasma attachment to HeLa cells occured (Table 16). The inhibition of ureaplasma attachment observed for cholesterol was not due to a permanent alteration of the ureaplasma membrane. Apart from adsorption of cholesterol from serum into the ureaplasma membrane there is little information in the literature regarding other serum components that may be adsorbed.

The results presented in this chapter indicate that to elucidate the attachment mechanism of ureaplasmas to eukaryotic cells, more information about the surface components of the ureaplasma membrane is needed. It appears that hydrophobic interactions may be involved but the exact mechanism of attachment remains unresolved.

CHAPTER VI DISCUSSION

This thesis describes certain interactions of U. urealyticum with N. gonorrhoeae and HeLa cells and extends the existing knowledge of certain features of U. urealyticum attachment to eukaryotic cells and how these may relate to the in vivo disease process. The disease-causing capabilities of a microorganism depend on the outcome of interactions between the host defense mechanisms and the pathogenic features of the microorganism. Smith (1977) states that a disease occurs when a microorganism progresses through the following four phases: entry into the host, multiplication in the host tissues, evasion of the host defenses and destruction of the host tissues. The results of the research in this thesis will be discussed in relation to these four phases of infection.

1. Entry into the host

1.1 Transmission

U. urealyticum has a very specific and reliable means of transfer from one host to the next since it is transmitted by sexual contact (McCormack et al 1972, Markham et al 1972, Taylor-Robinson & McCormack 1980a). McCutchan (1984) compared the dynamics of transmission for GU to those of NGU. The epidemiologically important events in venereal urethritis consist primarily of three intervals designated: the incubation period (the period of time from exposure to

onset of symptoms), the iatrotropic interval (the time from onset of symptoms to initiation of treatment) and the post treatment interval (from onset of treatment until the patient is no longer infectious) (McCutchan 1984). NGU is spread more effectively than GU because NGU has a longer incubation period, and iatrotropic interval (McCutchan 1984). The difference in disease severity combined with the longer incubation period likely explains why NGU can be overlooked in patients with GU. Fiumara and Calhoun (1982) emphasized the need for awareness of multiple, sexually transmitted diseases. Post-gonococcal urethritis (PGU) is non-gonococcal urethritis that develops after treatment of gonorrhoea (usually with a penicillin) (Csonka et al 1966, Markham et al 1972, Arya et al 1981). Approximately one-half of the men with gonorrhea subsequently developed PGU which was attributed to U. urealyticum or C. trachomatis (Csonka et al 1966, Markham et al 1972, Oriel et al 1975, Arya et al 1981). The results shown in Ch. III demonstrate that U. urealyticum can be co-isolated with N. gonorrhoeae in approximately one-third of the cases studied (Table 5). Both micro-organisms grow well together in vitro. The preliminary data reported in Ch. III showed that ureaplasmas may adhere to N. gonorrhoeae cells in vitro (Table 7). The attachment of ureaplasmas to N. gonorrhoeae (Table 7) was approximately four-times that observed for ureaplasmas to HeLa cells (Fig. 9). Such a cooperative association would enhance co-transmission in vivo.

Our results provide the first indication that M. hominis may be associated with alterations of the auxotype, plasmid content and Mr of P.I of N. gonorrhoeae (Table 9, Figs. 6, 7 & 8). Blake and Gotschlich (1983) reported that the P.I protein can move from the N. gonorrhoeae membrane into the membrane of red blood cells. The insertion of the P.I protein into the eukaryotic membrane has been postulated as the mechanism that may trigger uptake of the neisseria by eukaryotic cells (Blake & Gotschlich 1983, Young et al 1983). The auxotype and serum resistance of N. gonorrhoeae are also correlated with pathogenesis (Knapp & Holmes 1975, Schoolnik et al 1976, Sandstrom et al 1984). It would only be speculative to suggest that genital mycoplasmas affect pathogenisis or epidemiological studies of N. gonorrhoeae, nevertheless, the association of M. hominis with alterations of N. gonorrhoeae indicates that further research is warranted. Such a cooperative relationship would be beneficial to both of these fastidious microorganisms in an environment presumed to be nutrient-limiting (Smith 1977).

1.2 Attachment

Smith (1977) reported that entry into the host can take at least three forms: "attachment and multiplication without significant penetration; attachment to and penetration of mucosal cells in which they multiply with little or no spread from the initial site; and attachment and penetration into the underlying tissues". U. urealyticum appears to

belong to the first group. No convincing evidence has been published to indicate an intracellular location. I found that the infected HeLa cells had many long microvilli that were associated with the ureaplasmas (Fig. 20) and ureaplasmas were not observed inside the HeLa cells (Fig. 22). Although the distribution of microvilli changes with different stages of the cell cycle of uninfected HeLa cells (Lundgren and Roos 1976), microvilli of comparable length to those seen on ureaplasma-infected HeLa cells were seldom apparent on ureaplasma-free HeLa cells. The 10 nm to 15 nm gap observed between some of the attached ureaplasmas and HeLa cell membranes (Fig. 22) indicated that direct bridging was unlikely. It is possible that the gap that was observed was the junction of the glycocalyx of HeLa cells and the capsule of ureaplasmas. We showed that the ureaplasmas were at least partially protected from gentamicin when attached to HeLa cells (Table 12). Our data support the proposal by Mazzali and Taylor-Robinson (1971) that the eukaryotic cell may protect the ureaplasma from antibiotics.

The HeLa cell model used in this study was easy to use and the results obtained were reproducible. The number of attached ureaplasmas was time (Figs. 9, 12, 16 & 17) and inoculum (Fig. 23) dependent. Attachment of ureaplasmas to the HeLa cells was rapid, with about 5% of the ureaplasma inoculum attached after 1 hr (Figs. 9 & 12). Rapid attachment would be advantageous to ureaplasmas in vivo.

Brunius and Bolin (1983) reported that 6% of the Y. pseuodotuberculosis inoculum had bound to HeLa cells when held at 4°C for 1 hr. Ureaplasmas were always extracellular (Fig. 22) whereas Brunius and Bolin (1983) demonstrated that when incubated at 37°C Y. pseudotuberculosis was ingested by the HeLa cells.

The adhesins and receptors involved in the attachment of ureaplasmas to HeLa cells were not identified but several key features were apparent. By definition, the adhesin must be surface-located, thereby allowing interaction with the eukaryotic cell (Beachey 1981). For U. urealyticum strain 27 the predominant surface material that was iodinated appeared to be derived from horse serum components (Fig. 24). Initially, it was thought that horse serum components may have mediated attachment, but further studies showed that if serum components were involved, they could not be the sole adhesins because antiserum directed against horse serum did not inhibit attachment (Table 15). The horse serum associated with the ureaplasma membrane may act as a matrix that can rapidly dissociate in the presence of rabbit, horse or human serum (Fig. 11). The horse serum components adsorbed onto the ureaplasma membrane could mask the actual ureaplasma adhesins. Such a masking phenomenon by adsorbed serum components has also been suggested as an explanation for the loss of haemaglutination activity with older cultures of Mycoplasma gallisepticum (Bradbury & Jordan 1972). Masking of ureaplasma adhesins may explain

why specific antiserum raised against whole cells did not interfere with binding (Table 15).

Carbohydrates are another component on the surface of ureaplasmas that could be involved in attachment. The carbohydrate composition of the eukaryotic glycocalyx and the outer surface of the ureaplasma are similar. Robertson and Smook (1976) suggested that ureaplasmas have an affinity for the glycocalyx of eukaryotic cells. Although the proteinase K resistant material of ureaplasmas was immunogenic (Fig. 25), the role, if any, of carbohydrate in attachment remains to be determined.

2. Multiplication in host tissue

The in vitro model used in this study, like most other cell culture models (Mazzali & Taylor-Robinson 1971, Masover et al 1976a), provided evidence that ureaplasmas undergo limited replication and were only associated with HeLa cells for a short time (Fig. 9, Table 11). This is in contrast with the chronicity of infection seen in humans (Holmes et al 1974, Sautter & Brown 1980, McCutchan 1984) and the length of association reported in Fallopian tube explant models (Taylor-Robinson & Carney 1974) or animal models (Taylor-Robinson et al 1977, Kraus et al 1977). McGarrity (1985) has reported chronic ureaplasma infection of both HeLa and 3T-6 cell lines. He reported that the number of viable ureaplasmas increased over the incubation period. Indeed, when the infected monolayers were stained using the immunoperoxidase method there appeared to be "micro-

colonies" on the eukaryotic cells. Only McGarrity (1985) has reported multiplication of ureaplasmas in a eukaryotic cell culture model.

Four to six days post-infection fluorescent particles could be detected with FM (Figs. 13, 14 & Table 11) but no ureaplasmas grew on GM agar or in B broth. Other workers have reported that association with eukaryotic cells apparently altered ureaplasma characteristics (Masover et al 1976a, Shepard & Masover 1979, McGarrity 1985). How eukaryotic cells can alter ureaplasma characteristics is not known. Such alteration may explain the inability of myself and others to demonstrate multiplication of ureaplasmas in the cell culture models used.

3. Evasion of host defenses

This study did not directly address evasion of host defenses, but some characteristics that may be related were shown. To avoid host defenses ureaplasmas may use several mechanisms. Robertson et al (1984) demonstrated that all 14 serotypes produced IgA protease. Non-specific host defense mechanisms such as acidic pH and high urea effectively inhibit N. gonorrhoeae (McCutchin 1984) whereas U. urealyticum requires urea for growth and grows optimally at pH 6 (Shepard et al 1974). One feature that was not addressed in this study but which may be of importance, is the role of iron in the pathogenesis of ureaplasmas. Ureaplasmas colonize the urethra which is one of the major elimination routes for iron (0.1 mg/day in urine)

(Finkelstein et al 1983). Finkelstein et al (1983) have shown that the ability to compete with the host for iron is a major determinant of virulence and invasiveness for other microorganisms; its role in ureaplasma infection has not been assessed.

Masking of U. urealyticum specific antigens may be an evasion mechanism. Adsorbed horse serum components appear to have proper orientation to mask ureaplasma surface antigens since ureaplasmas appear to be coated with serum components which cannot be easily removed (Table 16). Gotschlich (1983) observed a similar "masking" for portions of the pili of N. gonorrhoeae. He found that the CB-2 fragment of N. gonorrhoeae pili was a constant region that mediates attachment to eukaryotic cells. However, the CB-3 fragment apparently masked the immunogenic potential of the CB-2 fragment. Whether the "capsule" reported by Robertson and Smook (1976) consists of adsorbed serum components or whether it is immunogenic is not known. By available assays the humoral antibody response to ureaplasmas in human serum is low (Bowie et al 1977a). This may reflect the generally poor immunogenicity of mycoplasmas (Fernald 1979). The host immune system may not recognize ureaplasmas as foreign because of the outer coating of host serum components. The apparent rapidity of dissociation shown by the ^{125}I studies reported in this thesis suggests that upon entering a human host the ureaplasmas could rapidly adsorb components from the new host, thereby avoiding recognition.

This phenomenon combined with their non-invasiveness may explain the low humoral antibody response to U. urealyticum. Studies designed to determine the degree of secretory antibody response would be of more relevance to NGU. McMillan et al (1979) could demonstrate no difference in the secrectory Ig A response in patients with GU, NGU or no disease. Whether this is related to the IgA protease activity of both N. gonorrhoeae and U. urealyticum is not known.

4. Damage of host tissues

The ability of U. urealyticum to damage host cells is controversial. The HeLa model used in this study and most other in vitro cell culture models (Mazzali & Taylor-Robinson 1971, Masover et al 1976, McGarrity 1985) showed no definite CPE. Although the lack of any CPE may be expected because of the short length of infection, McGarrity (1985) did not observe any CPE in chronic ureaplasma infections. The inability to produce defined CPE in the HeLa cell model may be a result of lack of virulence determinants of U. urealyticum propagated in vitro. Loss of virulence during in vitro passage has been well documented for many other pathogens (Smith 1977). Alternatively, the CPE produced may be host-dependent and would not be produced using in vitro models. U. urealyticum colonizes 30% to 50% of sexually active men without apparent damage (McCormack et al 1973). Approximately one-third of men with NGU (McCutchin 1984) have tissue damage that may be due to U. urealyticum. It

has been frequently suggested that some serotypes or biotypes may be more virulent than others, yet the data from serological studies do not support this (Shepard & Lunceford 1978, Robertson & Stemke 1985). Why some men who carry U. urealyticum develop NGU while others do not is not known.

5. Future Directions

This research provides evidence for previously unrecognized effects that genital mycoplasmas may have on N. gonorrhoeae. Further research is needed to determine if the mycoplasmas have a causal role in the auxotype, plasmid and P.I changes observed. Studies on nutritional cross-feeding and the effect of M. hominis products such as proteases and nucleases on the P.I of neisseria may be helpful. Further studies of urethral exudate would be useful to determine whether the interrelated growth of genital mycoplasmas and N. gonorrhoeae occurs in vivo and whether such an association affects epidemiological data that are based on the auxotype and serotype of neisseria isolates.

To understand the attachment process, knowledge of the role of major surface components is needed. For U. urealyticum the predominant surface features appear to be serum components which resist removal by extensive washing. Although many are aware of the problems associated with serum-adsorption by ureaplasmas in vitro, it would be of value to determine the role, if any, of such a phenomenon in virulence. Carbohydrate components have been implicated in

attachment and pathogenic characteristics of other microorganisms (Smith 1984b). Therefore, purified U. urealyticum lipoglycans should be used in competitive inhibition studies to determine if these are involved in the attachment process. Further research is necessary to establish whether or not the inability to detect ureaplasmas in the currently used attachment models is due to the inability of these microorganisms to grow on conventional media after association with eukaryotic cells. More sensitive and specific probes are needed to detect the ureaplasmas that have attached to eukaryotic cells. Dependence on viability assays, particularly indirect colour change methods, may be misleading. Mackowiak and Marling-Cason (1984) discussed the importance of comparing several in vitro assays when studying bacterial adherance. The advantages and limitations of the FM, CFU, and IR assays when used with the HeLa 229 cell model have been determined in this research; a stable, internal radiolabel would be a valuable tool in attachment studies.

In a discription of the evolutionary strategy of bacterial evasion of host defenses, Gotschlich (1983) points out: "If the range of the organism is limited to a single species or to a few very similar species, there is a strong negative selective pressure against those that are overwhelmingly virulent". U. urealyticum epitomizes the capability of low virulence. It survives well in the acidic environment of the genital tract and is effectively

transmitted from one host to another. NGU caused by U. urealyticum is more likely to be chronic than acute (Taylor-Robinson et al 1977). In many instances ureaplasmas are present without apparent disease (McCormack et al 1973). Studying the pathogenicity of ureaplasmas is difficult because of the subtle nature of infections caused by this organism. The results presented in this thesis provide a foundation for future investigations on the effect of genital mycoplasmas on Neisseria gonorrhoeae and characterization of adhesins and receptors involved in the attachment of Ureaplasma urealyticum to eukaryotic cells.

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APPENDIX I

1. Genital Mycoplasma agar

Genital mycoplasma (GM) agar is a minimal agar designed for culture of the genital mycoplasmas, M. hominis and U. urealyticum (Robertson 1978).

PPLO broth base without crystal violet (Difco Laboratories)	4.2 g
Agar No. 1 (Oxoid)	1.5 g
Yeast extract (Difco Laboratories)	0.2 g
N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (Hepes buffer) (Research Organics Inc., Cleveland, Ohio)	2.4 g
Distilled water	180.0 ml

The above ingredients were mixed, autoclaved at 120°C, under 15 lbs pressure for 15 mins and then cooled to 50°C and the following supplements added:

Pooled, normal-horse serum (Gibco Laboratories)	20.0 ml
Urea (Fisher Scientific Co.) (10% w/v sol.)	0.5 ml
GHL (Glycl-L-Histidyl-L-Lysine acetate) (Calbiochem Chemical Co.) (20 µg/ml)	0.2 ml
Ampicillin Sodium (Ayerst Laboratories)	2.0 ml

The pH was adjusted to 6.0 with sterile 2 N HCl. A sterile Cornwall syringe was used to dispense 5 ml aliquots into 60 mm sterile Petri dishes. The plates were allowed to dry at RT overnight and then stored at 4°C until needed. GM agar was used within one week of preparation. Those GM agar used for the CFU assays were used directly after drying overnight.

2. B Broth

B broth is an indicator broth designed for the detection of M. hominis and U. urealyticum (Robertson, 1978).

PPLO broth base without crystal violet (Difco Laboratories)	4.2 g
Bromothymol blue (Fisher Scientific Co.) (0.4% w/v)	2.0 ml
Yeast extract (Difco Laboratories)	0.2 g
Distilled water	180.0 ml

The above ingredients were mixed, autoclaved at 120°C, under 15 lbs pressure for 15 mins, cooled to 50°C and then the following supplements added:

Pooled, normal-horse serum (Gibco)	20.0 ml
Urea (Fisher Scientific Co.) (10% w/v)	0.5 ml
GHL (Glycl-L-Histidyl-L-Lysine acetate salt) (Calbiochem Chemical Co.) (20 µg/ml)	0.2 ml
Ampicillin Sodium (Ayerst Laboratories)	2.0 ml

The pH was adjusted to 6.0 with sterile 2 N HCl. This medium was stored at -20°C. The high concentration of ampicillin and the low pH are the basis for selectivity of B broth for ureaplasmas.

3. Standard broth

Standard broth is an indicator broth designed for the detection of Mycoplasmas.

PPLO broth base without crystal violet (Difco Laboratories)	2.1 g
Yeast extract (Difco Laboratories)	0.1 g
Glucose solution (Sigma Chemical Co.) (10% w/v)	1.0 ml
Phenol Red solution (Sigma Chemical Co.) (0.2% w/v)	1.0 ml
Distilled water	80.0 ml

The pH of the medium was adjusted to 7.8. The medium was autoclaved at 120°C for 15 minutes under 15 lbs pressure, cooled to 50°C and then supplemented with the following:

Pooled, normal-horse serum (Gibco)	20.0 ml
Ampicillin sodium (Ayerst Laboratories) (100 mg/ml)	1.0 ml

4. Standard Agar

Standard agar is a general purpose medium designed for the growth of a wide range of Mycoplasma species.

PPLO agar base (Difco Laboratories)	3.5 g
Yeast extract (Difco Laboratories)	0.1 g
Distilled water	80.0 ml

The agar was dissolved by heating and then the solution was autoclaved at 120°C for 15 mins under 15 lbs pressure. When the medium had cooled to 50°C, the following supplements were added:

Pooled, normal-horse serum (Gibco)	20.0 ml
Ampicillin sodium (Ayerst Laboratories) (100 mg/ml)	1.0 ml

The pH was adjusted to 7.8 using sterile 2N HCl. The Standard broth and the Standard agar were used for growth of mycoplasmas other than ureaplasmas.

5. **Auxotyping Media** (Dillon 1983)

These defined media were designed to test for nutritional deficiences which are then used to sub-group strains of N. gonorrhoeae. All of the chemicals used in the auxotyping media were reagent grade compounds that were obtained from the Sigma Chemical Co. The following description is excerpted from the auxotype preparation method of Dillon (1983).

5.1 Amino Acid Stock Solutions

All of the stock solutions were stored at -20°C until needed.

Amino Acid	Final Concentration (M)	Amount (g)	To dissolve add	Deionized water (ml)
L-citrulline	0.21	1.84	2 drops 10 N HCl	50
L-ornithine	0.21	1.39		50
L-proline	0.80	4.60		50
L-methionine	0.10	0.75		50
Mixture 1:				
Cysteine HCl	75 mM	0.59	2 drops	50
L-cystine	25 mM	0.30	10 N HCl	
Mixture 2:				
L-isoleucine	25 mM	0.66		
L-valine	25 mM	0.59		
L-leucine	25 mM	0.66	2 drops	200
L-threonine	25 mM	0.60	10 N HCl	
Mixture 3:				
L-serine	50 mM	0.26	1 drop	
L-alanine	0.1	0.45	10 N HCl	50
L-histidine	0.1	0.78		
Mixture 4:				
L-lysine	50 mM	0.73		
L-glutamine	0.1	1.46	2 drops	100
L-tryptophan	50 mM	1.02	10 N HCl	

5.2 Salts Solution

This solution was sterilized by filtration through 0.22 μm nitrocellulose filter and was stored at 4°C.

Chemical	Molarity	g/L
NaCl	1.0 M	58.44
K_2SO_4	50.0 mM	8.70
$MgCl_2.6H_2O$	20.0 mM	4.07
NH_4Cl	40.0 mM	2.15
disodium EDTA	0.1 mM	0.04

5.3 Other Solutions

These solutions were stored at -20°C.

a. Glutathionine-spermine;
1.55 g glutathione (0.1 M), and 1.0 g spermine (0.1 M) were made up to 50 ml with deionized water.

b. Uracil (0.2 M)
The uracil (1.12 g) was dissolved in 50 ml of 0.2 N NaOH.

c. Hypoxanthine (0.14 M)
The hypoxanthine (0.95 g) was dissolved in 50 ml of 0.2 N NaOH.

d. Cocarboxylase (TPP-HCl) (5 mM)
The cocarboxylase (0.021 g) was dissolved in 10 ml of deionized water.

e. Pyridoxal HCl (10 mL)
The pyridoxal (0.02 g) was dissolved in 10 ml deionized water.

f. Choline.Cl (50 mM)
The choline (0.069 g) was dissolved in 10 ml deionized water.

g. Biotin (0.6 mM)
The biotin (0.015 g) was dissolved in 100 ml deionized water.

h. $CaCl_2.2H_2O$ (0.125 M)
The $CaCl_2$ (1.84 g) was dissolved in 100 ml deionized water.

i. Ferric NH_4 citrate
The ferric NH_4 citrate (0.20 g) was dissolved in 50 ml deionized water.

j. Glycerol-lactate
9.2 g glycerol (2 M)
9.3 g DL-sodium lactate (60%) (0.5 M)
This solution was made up to 50 ml with deionized water.

5.4 Diluent

The diluent was used to prepare inocula of N. gonorrhoeae.

Component	Volume (ml)	Weight (g)
Salts solution	30.00	
Glycerol-lactate solution	1.50	
KH_2PO_4		0.42
K_2HPO_4		1.50

The above components were dissolved in sufficient deionized water to make a total volume of 300 ml. The pH was adjusted to 7.0 and then the solution was sterilized by filtration through a 0.22 µm nitrocellulose filter. This sterile solution was stored at 4°C until needed.

5.5 Double-Strength Basic Liquid Medium

The following formula was sufficient to prepare 1L of double-strength basic liquid medium. This medium was prepared on the day of use.

Components	Vol. (ml)	Weight (g)
Salts solution	200.0	
Deionized water	600.0	
KH_2PO_4 (10 mM)		3.5
K_2HPO_4 (30 mM)		8.2
$NaHCO_3$ (12 mM)		2.0
NaAcetate (25 mM)		6.8
Glucose (25 mM)		10.0
Glycerol-lactate solution	10.0	
Glutathione-spermine	5.0	
Amino Acid Mixture 2	20.0	
Amino Acid Mixture 3	5.0	
Amino Acid Mixture 4	10.0	
$CaCl_2$	1.0	
Ferric NH_4 citrate	1.0	
Vitamins; biotin	10.0	
cocarboxylase	1.0	
pyridoxal HCl	1.0	
choline	1.0	

The volume of the double-strength basic liquid medium was adjusted to 1 L and the pH was adjusted to 7.2 with 10N NaOH.

5.6 Double-Strength Agar

The agar solution was prepared on the day of use. Agarose (2.5 g) was added to 100 ml of deionized water in a 250 ml flask. The solution was autoclaved for 15 minutes under 15 lb/in^2 pressure and then cooled to 50°C in a water bath.

5.7 Auxotyping media

A series of eight beakers was labelled with the following symbols: POS (positive control), NEG (negative control), P, C, CO, M, U, H, (for proline, citrulline, ornithine, methionine, uracil and hypoxanthine, respectively). 100 mls of the double-strength liquid medium were added to each of the eight labelled beakers. The following solutions were added to each beaker, avoiding the specific beakers indicated.

Mixture 1	0.5 ml	to all except NEG
L-citrulline	0.5 ml	to all except C, CO
L-ornithine	0.5 ml	to all except CO
L-proline	0.5 ml	to all except P
L-methionine	0.5 ml	to all except M
Uracil	0.5 ml	to all except U
Hypoxanthine	0.5 ml	to all except H

There was enough double-strength basic liquid medium left over to test for two additional nutritional requirements. The complete solutions were separately sterilized by filtration through 0.22 µm nitrocellulose filters. Ten (100 x 15 mm) sterile Petri plates were

labelled with each of the following symbols: POS, NEG, P, C, O, M, U and H, and an orientation mark was placed on each plate. When the double-strength agar was 50°C, the double-strength basic liquid medium was warmed to 50°C for no longer than 15 mins. A flask of double-strength basic liquid medium was poured asceptically into a flask of double-strength agar and mixed by hand-rotation. The mixture was poured into the appropriately labelled plates and allowed to solidify at RT. After drying at RT for 24 hrs, complete sets of the auxotyping media were stored in plastic bags at 4°C. These plates could be stored for several months prior to use.

5.8 Interpretation of Results

Because all gonococci require cysteine (cystine can substitute for cysteine) for growth all of the N. gonorrhoeae strains should grow on the POS medium (the positive control) and no gonococcal isolate should grow on the NEG medium (the negative control). Each N. gonorrhoeae strain is tested for all 6 nutritional requirements. An isolate that fails to grow only on medium lacking proline is deficient in proline synthesis and its auxotype will be listed as P^-. The appearance of nine or fewer colonies or hazy or filmy zones of growth after 48 hrs incubation was recorded as negative.

6. H buffer

This non-nutritive buffer was designed for washing and suspending ureaplasmas. It was also used in the HeLa 229 cell model as the medium for short-term attachment experiments.

$MgCl_2$ (Fisher Scientific Co.)	0.2 g
NaCl (Fisher Chemical Co.)	5.8 g
N-2-hydroxyethyl piperazine-N´-2-ethanesulfonic acid (Hepes buffer) (Research Organics Inc.)	11.9 g

The solution was made up to 1 L and 2 N HCl was used to adjust the pH to 6.0. The medium was autoclaved at 120°C for 15 mins under 15 lbs pressure. In some instances, ampicillin (100 mg/ml) was added asceptically after the buffer had cooled.

7. Tissue Culture Medium (MEMS)

For routine propagation of HeLa cells, the following modification of MEM (Flow Laboratories) was used:

MEM without glutamine or sodium bicarbonate (Autopow) (Flow Laboratories)	1 x 1L pkt.
Ultra pure water Milli-Q water system (Millipore)	872 ml

This mixture was autoclaved at 120°C for 15 mins under 15 lbs pressure, cooled to RT and then the following supplements were added:

Glutamine (Sigma Chemical Co.) (0.2 M filter sterilized)	10 ml
$NaHCO_3$ (Sigma Chemical Co.) (7.5 % w/v filter sterilized)	15 ml
Fetal calf serum (Gibco) (heat inactivated at 56°C for 30 minutes)	100 ml
GHL solution (Glycl-L-Histidyl-L-Lysine acetate) (Calbiochem Chemical Co.) (20 µg/ml)	1 ml
Penicillin/Streptomycin (Sigma Chemical Co.) (100,000 U/ml and 100,000 µg/ml, respectively)	1 ml

The MEMS medium was filter sterilized through a 0.22 um nitrocellulose filter in a Stainless 142 mm Filter Holder (Millipore).

For some experiments ampicillin (100 mg/ml) was substituted for the penicillin/streptomycin solution and the medium was called MEMS-amp.

APPENDIX II

1. Specimen processing for transmission electron microscopy

The following solutions were prepared:

Glutaraldehyde: Glutaraldehyde (J.B. EM Services Inc., Pointe Claire, Que.) at a concentration of 50% (w/v) was diluted with 0.2 M cacodylate buffer pH 7.4 (J.B. EM Services Inc.) to prepare a 5% (w/v) stock. For use the stock solution was diluted 1:2 with distilled water to prepare a 2.5% solution.

Osmium fixative: Osmium tetroxide (J.B. EM Services Inc.) was used to prepare a 4% (w/v) osmium stock solution. This stock solution was diluted with 0.2 M cacodylate buffer to obtain a 1% (w/v) solution.

Uranyl acetate: Uranyl acetate (J.B. EM Services Inc.) was dissolved in distilled water to obtain a 4% (w/v) stock. This stock solution was diluted with 70% (v/v) ethanol to obtain a 1% (w/v) solution.

Embedding mixture:

Araldite 502 (Ernst F.Fullam Inc. Schenectady, N.Y.)	24.5 ml
DDSA (Fisher Scientific)	24.5 ml
DMP-30 (J.B. EM Services Inc.	1.0 ml

The above compounds were thoroughly mixed and then the mixture was stored at -20°C in plastic syringes prior to use. The specimens were prepared as follows:

Prefixation: Add 10% v/v of 2.5% glutaraldehyde fixative to the culture for the last hour of incubation (36°C).

Fixation (1): The cells were resuspended in 2.5% (w/v) glutaraldehyde fixative and held 1 to 4 hrs at 4°C.

Wash: The cells were washed five times in 0.1 M cacodylate buffer.

Fixation (2): The specimen was suspended in 1% (w/v) osmium fixative for 1 hr at RT.

Wash: The specimen was washed once in 0.1 M cacodylate buffer.

Post-fixation: The specimen was incubated in 1% (w/v) uranyl acetate for 1 hr at RT.

Dehydration: The specimens were processed through the following series of dehydration steps:

50% ethanol	20 min.
70% ethanol	20 min.
90% ethanol	20 min.
100% ethanol	20 min. X 3
propylene oxide	20 min. X 3

Embed: Propylene oxide (Eastman Kodak Corp, Rochester, N.Y.) and araldite were mixed in a proportion of 1:1 and the specimen was held in this mixture overnight at RT. The specimen was placed in araldite and incubated at 36°C for about 1 hr to remove air-bubbles and then was cured at 60°C overnight.

The specimen blocks were trimmed and then cut using a Reichert model OMUZ microtome equipped with a glass knife.

Sections producing silver-grey refraction of light were placed onto copper grids. These sections were stained with 2% (w/v) uranyl acetate for 20 mins and then with 0.2% (w/v) lead citrate(City Chemical Corp., N.Y.) for 5 min. The grids were dried and then inserted into a Philips 300 transmission electron microscope and examined at an accelerating voltage of 80 Kv.

2. Specimen processing for scanning electron microscopy

HeLa cells grown on 15 mm coverslips were processed for SEM by the following modification of the method reported by de Harven et al (1984).

Prefixation: Glutaraldehyde (0.1% w/v) was added to the culture for the last 15 min of incubation at 36°C.

Fixation: The specimen was placed in 1% (w/v) glutaraldehyde (prepared in PBS buffer pH 7.0) and held at RT for 1 hr.

Wash: The specimen was washed three times for 5 min each in PBS pH 7.0 containing 1% (w/v) immunoglobulin-free BSA (Sigma Chemical Co.) (PBS-BSA). The final wash was left on the specimen which was incubated overnight at 4°C.

1° antibody: The antiserum was diluted 1:100 in PBS-BSA. The specimen was incubated in the 1°-antibody solution for 2 hr at RT.

Wash: The specimen was washed three times for 20 mins each with PBS-BSA.

2° antibody: The antiserum was diluted in PBS-BSA to the appropriate concentration. The specimen was held in the 2°-antibody solution for 2 hr at RT.

Wash: The specimen was washed three times for 20 mins each with PBS-BSA.

Dehydration: The specimen was then processed at RT through the following series of dehydration steps:

50% ethanol	20 mins
70% ethanol	20 mins
90% ethanol	20 mins
100% ethanol	20 mins(repeated 3times)

3. Sodium dodecylsulfate polyacrylamide gel electrophoresis

3.1 Solutions for SDS-PAGE

3.1.1 Tris Buffer Preparation

The 1.875 M Tris HCl buffer was made as follows:

Trizma Base (Sigma Chemical Co.) was prepared by dissolving 22.7 g in 100 ml of distilled water. Trizma HCl (Sigma Chemical Co.) was prepared by dissolving 29.6 g in 100 ml distilled water. Trizma HCl was added to the 100 mls of Trizma base until the pH of the solution was 8.8.

The 1.0 M Tris HCl buffer was prepared as follows:

Trizma Base (Sigma Chemical Co.) was prepared by dissolving 12.1 g in 100 ml of distilled water. Trizma HCl (Sigma Chemical Co.) was prepared by dissolving 15.8 g in 100 ml distilled water. Trizma HCl was added to the 100 mls of Trizma base until the pH of the solution was 6.8.

3.1.2 Modified Laemmli's Running Buffer (Laemmli 1970)

The running buffer was prepared as follows:

Tris (Sigma Chemical Co.)	3.025 g
Glycine (Sigma Chemical Co.)	14.400 g
Sodium dodecyl sulfate (BDH Chemical Co.)	1.000 g

The above components were dissolved in sufficient ultra-pure water to a total volume of 1 L.

3.1.3 Modified Laemmli´s sample buffer (Laemmli 1970)

The sample buffer was prepared as follows:

Sodium dodecyl sulfate (BDH Chemical Co.)	2.000 ml
glycerol (Sigma Chemical Co.)	1.000 ml
1 M Tris pH 6.8	0.625 ml
Ultra pure water	6.350 ml
Bromophenol blue (Bio-Rad Laboratories Inc.) (1% w/v)	0.050 ml

The above compounds were mixed well, passed through a 0.22 µm nitrocellulose filter and then stored at RT.

3.1.4 Immunoblotting buffer:

The buffer used for "Western" electrophoretic transfer of proteins from SDS-PAGE to nitrocellulose sheets was prepared as follows:

Na_2HPO_4 (Sigma Chemical Co.)	11.5 g
NaH_2PO_4 (Sigma Chemical Co.)	4.6 g

Sufficient ultra-pure water was added to produce a total volume of 4 L.

3.2 Gel preparation:

The 12.5% separating gel was prepared as follows:

1.875 M Tris buffer pH 8.8	5.20 ml
acrylamide:bis (Bio-Rad Laboratories) (30:0.8 ratio)	10.800 ml
EDTA (Fisher Scientific Co.) 0.2 M	0.260 ml
Ultra pure water	9.450 ml
TEMED (Bio-Rad Laboratories)	0.013 ml

Ammonium persulfate (10% w/v) (Bio-Rad Laboratories)	0.260 ml

After mixing thoroughly, 25 ml of this solution was dispensed into each gel holder and water was used to overlay the gel. The gel was allowed to polymerize for about 15 mins.

The 5% stacking gel was prepared as follows:

acrylamide:bis (Bio-Rad Laboratories) (30:0.08 ratio)	2.500 ml
1 M Tris buffer pH 6.8	1.880 ml
EDTA (Fisher Scientific Co.) 0.2 M	0.150 ml
Ultra pure water	10.300 ml
TEMED (Bio-Rad Laboratories)	0.008 ml
Ammonium persulfate (Bio-Rad Laboratories) (10% w/v)	0.150 ml

The above compounds were thoroughly mixed. The water was poured off of the separating gel and approximately 6 mls of the stacking gel solution was dispensed ontop of the polymerized separating gel. The comb was inserted and, if necessary, more stacking gel solution was added. The gel was allowed to polymerize at RT for 45 mins.

4. Detection of carbohydrate

The sample was prepared in 50 µl of modified Laemmli's sample buffer as described for SDS-PAGE and then 10 µl of proteinase K (Boehringer Mannheim, Dorval, Que.) (1 mg/ml) was added. The sample was incubated for 2 hr at 60°C with agitation at 30 min intervals. The sample (10 µl) was

added to a lane of the 14% gel. After electrophoresis using standard SDS-PAGE conditions, the gel was reacted with a silver stain (Sect. 5).

5. Silver Stain

The following modification of the silver stain method of Tsai and Frasch (1982) was used in our study.

Solutions used for the silver stain:

5.1 Oxidizing reagent

The following compounds were mixed together:
1.4 g periodic acid (Sigma Chemical Co.) and 200 ml of a solution composed of 40% (v/v) ethanol and 7% (v/v) acetic acid (Sigma Chemical Co.).

5.2 Developer

To one liter of water was added 0.5 ml of a 37% formalin solution (Sigma Chemical Co.), and 50 mg of citric acid (Sigma Chemical Co.). The solution can be stored indefinitely at RT.

5.3 Silver Stain Solution

To 28 ml of 0.1 N NaOH was added 2 ml of concentrated NH_4OH (i.e. 29% (w/v) ammonium hydroxide solution) (Fisher Scientific Co.). The solution was mixed vigorously and 5 ml of 20% (w/v) $AgNO_3$ (Fisher Scientific Co.) was added slowly. A transient brown precipitate was observed. Finally, 115 ml of ultra-pure water was added.

5.4 Destain Solution

The destain solution consisted of 40% (v/v) ethanol and 5% (v/v) acetic acid.

The silver stain method is as follows:

The gel was fixed overnight in destain solution. (This step removes SDS which will give a murky yellow background to the gels). The dish was covered with Saran wrap. The destain solution was poured off and 200 mls of the oxidizing reagent was added and allowed to react, with mixing, for 5 mins. Using washed gloves, the gel was transfered to a second dish which contained 500 to 1000 ml of deionized water. The gel was washed three times with deionized water for 15 mins each. After the last wash the silver stain solution was added and allowed to react with the gel for 10 mins. The silver stain solution was poured off and the gel was washed three times, as before, with deionized water. The developer (about 400ml) was added and allowed to react until the bands observed were of the desired intensity or until the background began to yellow. Development was stopped by adding 7% acetic acid. The gel was stored in 7% acetic acid away from light because the bands fade upon prolonged exposure to the light.

7 Boxwood Cove,
Winnipeg, Manitoba
Canada
R2M 5K7
January 18, 1986

William M. McCormack (Editor)
Sexually Transmitted Diseases
Division of Infectious Diseases
Box 56, Downstate Medical Center
450 Clarkson Ave.
Brooklyn, New York 11203

Dear Dr. McCormack,

Some of my Ph.D. research results were published in the journal; Sexually Transmitted Diseases. I request permission to use, in my Ph.D. thesis (University of Alberta), the information and micrographs that were published in the following two articles:

1. Alfa, M.J., Robertson, J.A. (1984) The co-esistence of genital mycoplasmas and Neisseria gonorrhoeae isolated from the male urethra. Sex. Transm. Dis. 11:131-136.

2. Alfa, M.J., Chen, M. Robertson, J.A. (1985) Effects of Mycoplasma hominis on In vitro studies of Neisseria gonorrhoeae. Sex. Transm. Dis. 12:103-109.

Yours sincerely,

Michelle J. Alfa

Permission granted
provided that appropriate
reference to the original
publication is included
1-27-86